



The effects of magnesium administration on cardiac ventricular function, heart rate variability, and myocardial morphological changes in a chronic diabetes disease model in rats.

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Table of contents

Declaration	i
Acknowledgements	ii
Table of contents.....	iii
List of Figures and table	vi
List of Abbreviations	vii
Abstract	ix
Chapter 1: Introduction.....	1
Chapter 2: Literature review	3
2.1 Diabetes mellitus and cardiovascular disease	3
2.2 Diabetic cardiovascular complications and their underlying mechanisms	4
2.2.1 Diabetic cardiomyopathy.....	4
2.2.1.1 Mechanisms underlying diabetic cardiomyopathy	5
2.2.2 Coronary artery disease	8
2.2.2.1 Mechanisms underlying coronary artery disease in diabetes mellitus .	9
2.2.3 Diabetic cardiovascular autonomic neuropathy	10
2.2.3.1 The mechanisms underlying diabetic cardiovascular autonomic	
neuropathy.....	12
2.2.4 Electrolyte disturbances in diabetes	12
2.2.4.1 Mg ²⁺ and diabetes	13
2.2.5 Problem statement.....	14
2.2.6 Significance of the present study	15
Hypothesis	16
Aim of the study	16
Specific objectives	16
Chapter 3: Materials and methods	17
3.1 Ethical statement	17
3.2 Animals and animal care.....	17
3.3 Treatment protocol.....	18
3.4 In-vivo heart rate variability measurements.....	20
3.5 Heart isolation and perfusion technique.....	22
3.5.1 Cardiovascular function assessments	23

3.6 Histological studies	24
3.7 Western blot analysis	25
3.8 Plasma Mg ²⁺ assay	27
3.9 Chemicals and reagents	28
3.10 Statistical analysis.....	28
Chapter 4: Results.....	29
4.1 General parameters	29
4.1.1 Induction of diabetes and survival of the animals	29
4.1.2 The effect of Mg ²⁺ treatment on blood glucose level in STZ-induced diabetic rats	29
4.1.3 The effect of Mg ²⁺ treatment on the body weight changes in STZ- induced diabetes model	29
4.1.4 The effect of Mg ²⁺ treatment on the plasma Mg ²⁺ concentration in STZ - induced diabetic rats.....	31
4.2 Magnesium treatment improved heart rate variability in diabetes	32
4.2.1 The Time Domain parameters of HRV.....	32
4.2.2 The Frequency Domain parameter of HRV.....	34
4.2.3 The orthostatic stress response of heart rate.....	34
4.3 The effects of Mg ²⁺ and STZ treatment on cardiac synaptophysin protein expression.....	35
4.4 The effects of Mg ²⁺ and STZ treatment on cardiac mitochondrial ATP5A expression.....	36
4.5 The effects of Mg ²⁺ treatment on ECG changes in STZ-induced diabetic model	37
4.6 The effects of Mg ²⁺ treatment on haemodynamic parameters in STZ-induced diabetic rat model.....	39
4.6.1 Mg ²⁺ treatment improved hemodynamic function.....	39
4.6.2 The effects of STZ and Mg ²⁺ treatment on coronary flow rate.....	41
4.7 The effects of STZ and Mg ²⁺ treatment on cardiac structure.....	41
4.7.1 Changes in heart weight	41
4.7.2 Analysis of cardiac cell size	42
4.7.3 Analysis of ventricular tissue fibrosis	43
Chapter 5: Discussion	46
References.....	52
Appendices	79
Appendix 1: Animal Ethics Committee approval letter	79

Appendix 2: STZ preparation	80
Appendix 3: Histological protocol	81
Appendix 4: Western blot protocol	85

List of Figures and table

Figure 1: Summary diagram of the experimental protocol.....	20
Figure 2: The technique of the HRV recording.	22
Figure 3: The isolated rat heart mounted on the Langendorff perfusion system.	24
Figure 4: Weekly measurement of blood glucose and body weight for the different treatment groups..	30
Figure 5: Plasma Mg^{2+} Concentration for the different treatment groups.....	31
Figure 6: The effects of Mg^{2+} and STZ treatment on the time domain parameter of HRV and HR for the various treatment groups.	33
Figure 7: The effects of Mg^{2+} and STZ treatment on frequency domain parameter for the different treatment groups.	34
Figure 8: The effects of STZ and Mg^{2+} treatment on the response of HR to orthostatic stress test.	35
Figure 9: Representative immunoblot bands of synaptophysin and alpha tubulin proteins for the various treatment groups.....	36
Figure 10: Representative immunoblot bands for ATP5A and alpha tubulin proteins for the different treatment groups..	37
Figure 11: The effects of Mg^{2+} and STZ treatment on ECG waveforms.	38
Figure 12: The effects of STZ and Mg^{2+} treatment on haemodynamic parameters for various treatment groups.....	40
Figure 13: The effects of STZ and Mg^{2+} treatment on coronary flow rate for different treatment groups.	41
Figure 14: The effects of STZ and Mg^{2+} treatment on heart weight/ body weight ratio.	42
Figure 15: The effects of STZ and Mg^{2+} treatment on histology of cardiomyocyte... ..	43
Figure 16: The effects of Mg^{2+} and STZ treatment on the extent of interstitial fibrosis for different treatment groups.	44
Figure 17: The effects of STZ and Mg^{2+} treatment on the extent of perivascular fibrosis for the various treatment groups.	45
Table 1: Summery data of ECG characteristics for various treatment groups.....	39

List of Abbreviations

AGEs – Advanced glycation end products

ANOVA – Analysis of variance

ATP – Adenosine triphosphate

Ca²⁺ ion – Calcium ion

CAD – Coronary artery disease

CVD – Cardiovascular disease

DCAN – Diabetic cardiac autonomic neuropathy

DCM – Diabetic cardiomyopathy

DM – Diabetes mellitus

ECG – Electrocardiograph

ECL – Enhanced Chemiluminescence

H&E – Haematoxylin and Eosin

HRV – Heart rate variability

HRP – Horseradish peroxidase

HR – Heart rate

K⁺ ion – Potassium ion

K-H – Krebs-Henseleit

LV – Left ventricle

LVDP – Left ventricular developed pressure

Mg²⁺ ion – Magnesium ion

MgSO₄ – Magnesium sulfate

MI – Myocardial infarction

PBS-T – Phosphate buffered saline-tween

PVDF – Polyvinylidene fluoride

RIPA – Radioimmunoprecipitation assay

ROS – Reactive oxygen species

RPP – Rate pressure product

SA – South Africa

SDS – Sodium dodecyl sulphate

STZ – Streptozotocin

USA – United States of America

Abstract

Introduction: Diabetes mellitus (DM) is a leading cause of morbidity and mortality all over the world, and the main cause of the mortality is cardiovascular complications. Such diabetic cardiovascular complications include coronary heart disease, cardiac autonomic neuropathy and ventricular dysfunction. Furthermore, DM is associated with electrolyte disturbances such as those involving potassium, calcium and magnesium (Mg^{2+}). Among these electrolyte disturbances hypomagnesemia is common in diabetes and is associated with increased cardiovascular risk. Recent evidence has shown that Mg^{2+} supplementation can prevent cardiac autonomic dysfunction and improve ventricular compliance in acute DM. However, the underlying mechanisms of Mg^{2+} action and Mg^{2+} effects in chronic DM are unknown. Therefore, the present study explored the effects of Mg^{2+} administration and its possible mechanisms of action in chronic streptozotocin (STZ) induced diabetic rats.

Methods: Adult male Wistar rats were injected intraperitoneally (i.p) once with either STZ (50 mg/Kg body weight) or the STZ vehicle (citrate buffer). The rats were then injected i.p once daily with either magnesium sulphate ($MgSO_4$; 270 mg/Kg body weight) or the $MgSO_4$ vehicle (normal saline) for 28 consecutive days. Blood glucose and body weight were measured throughout the period of the study. On day 28 of the experiments, in-vivo heart rate variability (HRV) parameters were measured to assess cardiac autonomic function using tail pulse plethysmography. Orthostatic stress was induced by tilting the animals from flat position to 70° head-up position. Ex-vivo hemodynamic and electrocardiograph (ECG) measurements were performed on a Langendorff perfusion system. Histological studies of ventricular tissue were performed using haematoxylin-eosin and Masson's trichrome staining. Western blot analyses of the cardiac autonomic presynaptic marker (synaptophysin) and of the mitochondrial marker of oxidative stress (ATP5A) were performed on right atrial tissue. Plasma Mg^{2+} concentration was measured using automated photometric assays.

Results: STZ treatment significantly increased the blood glucose level and decreased the body weight, and these STZ effects were not prevented by Mg^{2+} treatment. Diabetes decreased the root mean square differences of successive normal-to-normal intervals (RMSSD) and increased the low frequency (LF) /high frequency (HF) power ratio, which are both indicative of abnormal HRV. These diabetes effects on HRV parameters were significantly prevented by Mg^{2+} treatments ($P < 0.05$, STZ+Mg vs. STZ). DM also reduced both the heart rate and orthostatic stress-induced tachycardia, and these effects were reversed by Mg^{2+} treatment ($P < 0.05$, STZ+Mg vs. STZ).

DM also decreased the left ventricular (LV) developed pressure and the maximal rate of LV pressure increase (+dP/dt), and these diabetic effects were prevented by Mg^{2+} treatment ($P < 0.05$, STZ+Mg vs. STZ). DM also decreased the maximal rate of LV pressure decline (-dP/dt) and the rate pressure product, but these parameters were not improved by Mg^{2+} treatment. DM and Mg^{2+} treatment did not affect the ECG waveforms and the coronary flow rate in all groups. Histologically, there were no differences in ventricular cardiomyocyte width or in the extent of interstitial fibrosis in all groups. Western blot analysis qualitatively showed a decrease in the expression of synaptophysin in DM that was prevented by Mg^{2+} treatment. Neither DM nor Mg^{2+} treatment altered ATP5A expression. The plasma Mg^{2+} concentration was not altered by DM or Mg^{2+} treatment.

Conclusion: This study showed that Mg^{2+} treatment prevented cardiac autonomic dysfunction and improved hemodynamic function impairment in chronic DM. Based on the expression of synaptophysin, the mechanism through which Mg^{2+} improved cardiac autonomic function could involve the prevention of synaptic degradation in diabetes. The effects of Mg^{2+} on hemodynamic impairment in diabetes seemed to be unrelated to the Mg^{2+} effects on the cardiac histological structure or on the changes in coronary perfusion. Moreover, the overall effects of Mg^{2+} in diabetes were independent of its effects on the blood glucose level or the alteration of plasma Mg^{2+} level. Thus, Mg^{2+} treatment may have long-lasting therapeutic effects on ventricular dysfunction and cardiac autonomic impairment in chronic diabetes, but further studies are needed to explore the precise underlying mechanisms.

Chapter 1: Introduction

Diabetes mellitus (DM) is a rapidly growing metabolic disorder world-wide and is a one of the main leading causes of morbidity and mortality (Guariguata et al. 2011). DM affected 382 million adults world-wide by 2013, and is projected to increase to 592 million by 2035 (Guariguata et al. 2014). Therefore, because of its increased incidence globally, DM can be considered an epidemic chronic disease (Wild et al. 2004). In Africa, the number of diabetic patients in 2010 was 12.1 million and is projected to rise to 23.9 million by 2030 (Hall et al. 2011). Furthermore, the prevalence of DM in developing countries like South Africa is currently approximately 9% (Bertram et al. 2013), and projected to reach approximately 69% by 2030 (Shaw et al. 2010). As a result, DM creates substantial economic burden in many regions, especially in the African continent. The economic burden of DM in the developing countries is mainly related to the cost of disease management and patients' disability, both of which result in loss of productivity in the community (Kirigia et al. 2009).

DM is a metabolic condition in which there is insulin deficiency, insulin resistance or both. The pathogenic processes involved in DM include the destruction of insulin-producing beta cells of the pancreas and resistance to insulin action at cellular level that lead to hyperglycaemia. In addition to hyperglycaemia, insulin deficiency also leads to other abnormalities in the metabolism of lipid and proteins, all of which have adverse effects on tissues (Alberti and Zimmet 1998, Kerner and Bruckel 2014).

Globally, DM is considered the fifth leading cause of death, following the other common conditions such as infectious diseases and cancer (Roglic et al. 2005). The main causes of death in DM include acute life-threatening complications such as hypoglycaemia, hyperglycaemia with ketoacidosis or non-ketotic hyperosmolar conditions, as well as long-term complications that involve multiple body systems. The multi-system complications include cardiovascular diseases (CVD), eye, renal problems, gastrointestinal, and neurological damage. Among these complications, CVD account for nearly half of diabetic complications (Hobbs 2006, Rao Kondapally Seshasai et al. 2011). In addition, most of diabetic morbidity and mortality are due to

CVD (Soedamah-Muthu et al. 2006). Diabetic patients with CVD have a poorer prognosis than diabetic patients without CVD (Smith, Marcus, and Serokman 1984). Moreover, pathological and epidemiological studies have shown that DM is an independent risk factor for CVD (Wilson et al. 1998).

Diabetic cardiovascular complications include coronary artery disease (CAD), diabetic cardiac autonomic neuropathy (DCAN), diabetic cardiomyopathy (DCM), and other cardiac degenerative conditions. According to American Heart Association, DM is the main risk factor for CVD besides hypertension, dyslipidaemia, and smoking (Grundy et al. 1999). Diabetic CVD occur as a result of metabolic, structural, and electrical changes in the heart and blood vessels. These derangements cause cellular changes that leads to pathological remodelling in the cardiovascular tissue (Eguchi et al. 2012). The pathological remodelling process is further compound by disturbances in electrolytes that are common in diabetes, such as those involving calcium (Ca^{2+}), potassium (K^{+}), and magnesium (Mg^{2+}).

Electrolyte disturbances in DM have been linked to the impairment of excitation-contraction coupling of the heart (Marks 2003), arrhythmogenesis (Del Gobbo et al. 2012), and atherosclerosis (Atabek et al. 2006). Among these electrolyte disorders, hypomagnesemia is frequent in patients with DM (Pham et al. 2007). Furthermore, DM is an independent risk factor of hypomagnesemia in elderly patients (Liamis et al. 2013). In addition, hypomagnesemia has been implicated in the pathogenesis of diabetic complications, including CVD (Chetan P. Hans 2002).

Mg^{2+} treatment in DM has been proposed to improve blood glucose level, oxidative stress damage, insulin sensitivity, and prevent development of diabetic complications (Barbagallo et al. 1999, Nagai and Ito 2013, Rodriguez-Moran and Guerrero-Romero 2003). Moreover, a recent study in acute diabetic model shown that Mg^{2+} treatment attenuates cardiac autonomic dysfunction and improves ventricular compliance (Amoni et al. 2016). These Mg^{2+} effects were shown to be independent on its effects on hyperglycaemia, hyperlipidaemia, and were unrelated to changes in plasma or tissue Mg^{2+} levels. However, these Mg^{2+} effects remain unclear whether they are sustained in chronic DM. In addition, the underlying mechanisms are unclear.

Chapter 2: Literature review

2.1 Diabetes mellitus and cardiovascular disease

DM is a complex metabolic disorder characterized by impaired production of insulin or resistance to insulin action at cellular level. Typically, diabetes is diagnosed when fasting blood glucose ≥ 7 mmol /l, random plasma glucose ≥ 11.1 mmol /l, glycated haemoglobin A1c (Hb A1c) ≥ 48 mmol /mol or oral blood glucose tolerance test ≥ 11.1 mmol /l (Association 2014, Craig, Hattersley, and Donaghue 2009). According to American Heart Association (2014), DM is classified mainly into two types: insulin-dependent DM (type 1) and non-insulin dependent DM (type 2). Type 1 DM constitute 5-10 % of diagnosed diabetic patients, and mainly occurs due to autoimmune destruction of pancreatic beta cell. About 90-95 % of diabetic patients belong to type 2 DM, which is mainly due to insulin resistance. In type 2 DM, multiple genetic factors and obesity have been linked to insulin resistance and beta cell defects (Alberti and Zimmet 1998, Rayburn 1997).

Several metabolic derangements in DM predispose to cardiovascular complications. Hyperglycaemia mediates cellular injury through the activation of alternative glycolysis biochemical pathways. Such pathways include polyol pathway flux, increased hexosamine pathway flux, and activation of protein kinase C isoforms. These alternative biochemical pathways lead to impairment of cellular energy utilization and cardiovascular function (Davidoff et al. 2004, Mokuda et al. 1990, Robertson 2004). There is also hyperglycaemia- induced mitochondrial overproduction of free radicals, which in turn induces cardiovascular tissue damage due to oxidative stress (Brownlee 2001). Hyperglycaemia also leads to the formation of advanced glycation end-products (AGEs), which interfere with cardiovascular tissue integrity. DM also causes electrolyte disorders and acid-base disorders such as ketoacidosis, lactic acidosis, and hyperchloremic acidosis (Sotirakopoulos et al. 2012). Moreover, DM induces dyslipidaemia where there is elevated triglycerides, decreased high density lipoprotein, and increased low density lipoprotein (Taskinen 2003). Dyslipidaemia also leads to oxidation of lipoproteins and formation of pro-atherogenic glycoxidation products (Lopes-Virella et al. 2005).

2.2 Diabetic cardiovascular complications and their underlying mechanisms

2.2.1 Diabetic cardiomyopathy

Diabetic cardiomyopathy (DCM) is a disease condition caused by DM-induced structural and functional abnormalities in the myocardium that occur independently of other contributing factors such as CAD or hypertension. DCM is a leading cause of heart failure (Hayat et al. 2004), whereas DM is an independent risk factor for heart failure. The evidence supporting these findings has been reported in several studies, including the Framingham Study (Kannel, Hjortland, and Castelli 1974), Cardiovascular Health Study (Gottdiener et al. 2000), and United Kingdom Prospective Diabetic Study (Stratton et al. 2000). Clinically, DCM can be assessed by non-invasive techniques such as tissue doppler echocardiography, which can detect the abnormality even before the onset of symptoms, or by invasive techniques such as cardiac catheterization (Hayat et al. 2004).

The structural changes in DCM include left ventricle (LV) hypertrophy and myocardial fibrosis. LV hypertrophy is a consequence of increased LV mass and leads to ventricular stiffness and LV dysfunction (Devereux et al. 2000, Levy et al. 1990). The Strong Heart Study (Devereux et al. 2000) has reported that diabetic patients have increased LV mass, wall thickness, and arterial stiffness.

Cardiac diastolic dysfunction is an initial sign of functional changes in DCM (Karamitsos et al. 2007, Raev 1994). Diastolic dysfunction is widely reported in diabetic patients (Bouchard et al. 1989, Rajan and Gokhale 2002). Furthermore, experimental studies have documented the occurrence of diastolic dysfunction in diabetic animal models through echocardiographic measurements (Semeniuk, Kryski, and Severson 2002) and isolated heart perfusion experiments (Aasum et al. 2003). It has been observed that the diastolic dysfunction in DCM precedes systolic impairments in diabetic patients and diabetic animal models (Litwin et al. 1990, Schannwell et al. 2002). Furthermore, the occurrence of both diastolic dysfunction and systolic dysfunction seems to be related to the duration of DM (Shapiro, Howat,

and Calter 1981). In addition, the signs of ventricular dysfunction have been observed in type 1 DM than in type 2 DM (Lavine 1999).

2.2.1.1 Mechanisms underlying diabetic cardiomyopathy

The pathogenic mechanisms of DCM are multifactorial. These mechanisms include metabolic disturbances, oxidative stress, cardiac remodelling, mitochondrial dysfunction, and others (Spector 1998, Tziakas, Chalikias, and Kaski 2005).

Hyperglycaemia is also a key factor that underlies several metabolic disturbances in DCM (Chatham and Seymour 2002). Hyperglycaemia mediates its pathogenic effects via several mediators such as oxidative stress and increased formation of AGEs.

2.2.1.1.1 Oxidative stress

An increase in oxidative stress in the diabetic heart is the main factor in the development and advancement of DCM (Cai 2006, Cai et al. 2006, Crespo et al. 2008). Oxidative stress is a state of imbalance between pro-oxidant and antioxidant mechanisms leading to tissue damage (Sies 1997). The oxidative stress occurs due to increased production of free radicals such as the reactive oxygen species (ROS) and the reactive nitrogen species. The production of free radicals in DM occurs through glucose auto-oxidation (which is the main source of free radical production), the formation of AGEs, and mitochondrial damage (Bonnetfont-Rousselot 2002, Ceriello and Motz 2004).

The mitochondrion is the main source of ROS in DM (Nishikawa et al. 2000). The mitochondrion is the organelle responsible for providing the cell with energy via the process of oxidative phosphorylation (Neubauer 2007). The mitochondrial adenosine triphosphate (ATP) is the main source of energy for cellular metabolism (Schapira 2006). The ATP production in the mitochondria occurs under activity of ATP synthase (F₀F₁ complex), which is located in the mitochondrial membrane and consist of two subunits F₀ and F₁. The F₁ subunit consists of five subunits (α , β , γ , δ , and ϵ), where the β subunit is the catalytic site of the enzyme, while the α subunit is the functional nucleotide of the enzyme (Abrahams et al. 1994). DM causes changes in the structure and function of mitochondria (Pierce and Dhalla 1985, Shen

et al. 2004). This mitochondrial disturbances result in reduction of ATP production due to defect in enzymatic activity such as ATP synthase, therefore leading to cardiac dysfunction (Pierce and Dhalla 1985). Additionally, a previous study has shown a decrease in the ATP synthase activity in diabetic heart that could be prevented by metallothionein (Cong et al. 2014).

DM is also associated with compromised antioxidant defences mechanisms. Antioxidant mechanisms include both enzymatic and non-enzymatic components that work together to remove free radicals. The enzymatic antioxidants include glutathione peroxidase and superoxide dismutase (Baynes 1991, Baynes and Thorpe 1999, Saxena et al. 1993), whereas non-enzymatic components include vitamins. The study by Patel and colleagues showed that there is a reduction in the activity of antioxidant enzymes such as superoxide dismutase in the diabetic heart with ventricular dysfunction (Patel, Raghunathan, and Porwal 2014). Therefore, the alteration in the activity of antioxidant defence leads to oxidative stress and cell injury in DM (Maritim, Sanders, and Watkins 2003).

2.2.1.1.2 Formation of AGEs

The formation of AGEs in DM plays a part in the pathogenesis of DCM. The AGEs are a group of varied compounds that arise from series irreversible non-enzymatic reactions between glucose and proteins (Basta, Schmidt, and De Caterina 2004). DCM is characterized by an increase in the deposition of collagen and AGEs in-between cardiac myofibers, leading to interstitial fibrosis, and around cardiac blood vessels leading to perivascular fibrosis. The deposition of collagen and AGEs in the heart leads to stiffness of the myocardium, ventricular dysfunction, and eventually heart failure (Deluyker, Evens, and Bito 2017, Regan et al. 1977, van Heerebeek et al. 2008). The production of AGEs also causes cellular dysfunction through modification of intracellular and extracellular proteins. In addition, AGEs decrease endothelial production of nitric oxide, and the reduced bioavailability of nitric oxide increases LV diastolic stiffness (Bucala, Tracey, and Cerami 1991, Heymes et al. 1999).

AGEs also bind to specific AGE receptors (called RAGE), which are expressed on the cell surfaces of cardiac, vascular, and inflammatory cells (Brett et al. 1993,

Goldin et al. 2006, Schmidt et al. 1999). The activation of RAGE induces the production of ROS (Yan et al. 1994) as well as the modulation of transforming growth factors and fibrosis (Striker and Striker 1996). DM induces an increase in the expression of RAGE in the cardiomyocyte that leads to modulation of nuclear factors and induction of cardiac dysfunction (Aragno et al. 2006). Ma et al.(2009) showed that the modulation of RAGE expression prevents diabetes-induced contractile dysfunction in diabetic model. The AGEs-RAGE interaction also leads to reduced enzymatic activity of sarcoplasmic reticulum in the diabetic heart, and participate to impairment in cardiac function (Arai 2002, Bidasee et al. 2004).

2.2.1.1.3 Cardiac remodelling

Cardiac remodelling is an important pathogenic factor for DCM and other cardiac disturbances. Remodelling refers to alteration in the structure and electrical function of the heart. Ventricular structural remodelling occurs as a result of cellular, molecular, and interstitial changes in response to cardiac disease or injury (Cohn, Ferrari, and Sharpe 2000). The changes in cardiac structure or morphology manifest as cardiac cell hypertrophy, cardiac fibrosis, and cell death (Swynghedauw 1999). Concentric LV hypertrophy is frequently detected in diabetic patients (Bella et al. 2001, Devereux et al. 2000), where an increase in the myocardial mass is attributed to an increase in cardiomyocyte size. In addition, cardiac fibrosis is a feature of LV hypertrophy due to alteration of extracellular matrix. The main collagen deposition detected in cardiac fibrosis is collagen type I and III, which lead to impairment of ventricular contraction and relaxation (Weber 1997). In DM, the specific factors that may be involved in cardiac remodelling include oxidative stress and the generation of AGE by-products. Oxidative stress leads to ventricular remodelling through several responses, including the modulation of extracellular matrix (King et al. 2003), alteration of signalling pathways that lead to cardiac hypertrophy, and apoptosis (Cesselli et al. 2001, Takano et al. 2003).

Diabetic structural remodelling is also compounded by electrolyte disturbances and electrical remodelling. DM is associated with cardiac contractile dysfunction due to disturbance in the Ca^{2+} transport system (Ganguly et al. 1983, Luo and Anderson 2013). Several studies have shown through in-vivo and ex-vivo experiments in diabetic rat models that there is an alteration in the Ca^{2+} homeostasis and Ca^{2+}

signaling in DM (Choi et al. 2002, Ren and Bode 2000, Teshima et al. 2000). A consequence of reduced Ca^{2+} content of sarcoplasmic reticulum is the decreased release of Ca^{2+} during ventricular systolic period (Bouchard and Bose 1991, Lagadic-Gossmann et al. 1996). In addition, Trost et al. (2002) found that the increased expression of sarcoplasmic reticulum Ca^{2+} pump could protect the diabetic heart from contractile dysfunction. The other aspect related to Ca^{2+} that contributes to contractile dysfunction in DM is an alteration in the contractile proteins such as the activity of Ca^{2+} ATPase (Dillmann 1980, Takeda et al. 1996). Hamblin et al. (2007) also demonstrated that the impaired cardiac function in chronic diabetic rat model was associated with alteration in the expression of several cardiac proteins such as myosin and is accompanied with an increase in oxidative stress.

DM also induces electrolyte imbalances and alteration in the electrical properties of the cardiomyocyte. Such changes comprise cellular ion homeostasis and the generation of normal action potentials (Jourdon and Feuvray 1993, Shimoni et al. 1994). The ionic alterations in cardiac remodelling involve complex disturbance in the function and expression of the ion currents (K^+ , Ca^{2+} and sodium). These alterations in electrolytes impair cardiac repolarization and impair the heart rhythm (Akar and Rosenbaum 2003, Cutler, Jeyaraj, and Rosenbaum 2011). Specifically, diabetic electrical remodelling of the atrium leads to interatrial conduction defects that predispose to atrial fibrillation (Kato et al. 2006), whereas ventricular electrical remodelling leads to life threatening arrhythmia such as ventricular tachycardia and fibrillation (Coronel et al. 2013, Wang and Hill 2010).

2.2.2 Coronary artery disease

CAD is a spectrum of conditions caused by pathology in the coronary arteries of the heart, and includes myocardial infarction (MI) and angina. DM is independent risk factor for CAD (Koskinen et al. 1992). The early diagnosis of CAD in diabetic patients is important in order to achieve appropriate and timely treatment. In order to promote the early detection, the American Diabetes Association (1998) has established screening programmes for CAD in diabetic patients. These programmes included old age patients with high blood pressure, and increased lipid profile as well as smokers. The diagnostic tools used for these evaluations include resting and

exercise electrocardiograph (ECG), echocardiography, and perfusion imaging techniques.

DM increases the incidence of MI by two to three times, and MI in diabetic patients have poorer prognosis than in non-diabetic patients (Grundy et al. 1999, Herlitz et al. 1988). CAD is a leading cause of mortality in patients with DM and accounts for nearly 80% of all diabetic mortality (Thom et al. 2006). Despite the advances in medical evaluation of CAD, the diabetic patients with CAD have a poor prognosis post MI than that in non-diabetic patients. In addition, diabetic patients with MI have a higher risk of re-infarction, angina, arrhythmia, and heart failure than in non-diabetic subjects (Abbott et al. 1988, McGuire et al. 2000). It has been reported by Kahn et al. (2012) that the mortality rate is higher in patients with DM following percutaneous coronary artery intervention than that in non-diabetic patients. Moreover, the extent of coronary arteries involvement is higher in DM, and diabetic patients have a higher incidence of multi-vessel disease compared to non-diabetic patients (Waller et al. 1980).

2.2.2.1 Mechanisms underlying coronary artery disease in diabetes mellitus

There are several pathogenic factors predisposing to atherosclerotic CAD in DM. Lipid abnormalities are an important atherogenic risk factor (Bakogianni et al. 2001), and are significantly related to glycaemic control. Abnormalities of lipid profile in DM include elevated triglycerides, increased concentration of low density cholesterol, and decreased concentration of high density cholesterol. Hyperlipidaemia leads to lipid peroxidation of low density lipoprotein through superoxide-dependant pathways that cause free radical production (Kawamura, Heinecke, and Chait 1994). In addition, there is an increase in lipid peroxidation by-products such as malondialdehyde, which is the principle product of polyunsaturated fatty acid peroxidation and has been identified in the serum of diabetic patients. Increased levels of malondialdehyde are also associated with the advancement of diabetic CAD (Dierckx et al. 2003, Gallou et al. 1993). Dyslipidaemia, together with hyperglycaemia and insulin resistance also induce dysfunction of vascular endothelial and smooth muscle cells (Renard et al. 2004, Suzuki et al. 2001, Williams et al. 1996). Endothelial dysfunction in DM is associated with low levels of

nitric oxide and increased levels of endothelin 1 (Eren, Yilmaz, and Aydin 2013). The endothelial dysfunction in DM impairs vasodilation due to decreased production of vasodilators such as nitric oxide through the inhibition of enzymes involved in the synthesis of the vasodilators (Hink et al. 2001). Inflammation is also an important pathogenic factor for atherosclerosis in DM, because of an increase in the production of inflammatory and pro-inflammatory cytokines (Pickup 2004). In addition, disturbances in platelet function and abnormality in coagulation factors contribute to CAD pathogenesis (Mansfield, Heywood, and Grant 1996, Vinik et al. 2001). Furthermore, hyperglycaemia-induced oxidative damage and formation of AGEs have been implicated in atherosclerosis in diabetic patients and the concentration of AGEs increase with severity of atherosclerosis (Nakamura et al. 1993).

2.2.3 Diabetic cardiovascular autonomic neuropathy

DCAN is a frequent form of neuropathy in DM, and it occurs in one fourth of type 1 DM and in one third of type 2 DM patients (Ziegler 1994). Although the prevalence of DCAN is increased both with age and with the duration of DM (Low et al. 2004), DCAN can also be discovered at the first time of diabetic diagnosis (Vinik, Freeman, and Erbas 2003). DCAN is a cause of increased morbidity and mortality in the DM because it is accompanied by an increase in the occurrence of silent myocardial infarction, cardiac dysrhythmias, and sudden death (Kempler et al. 2002, Ziegler 1994). In addition, DCAN increases risk of mortality in diabetic patients with previous MI (Miettinen et al. 1998).

The cardiac autonomic nervous system plays a key role in regulating the changes in cardiac rhythm and the speed of electrical propagation in the cardiac conduction system. Autonomic cardiac innervation includes sympathetic and parasympathetic systems. The sympathetic innervation originates from spinal cord thoracic segments, whereas the parasympathetic innervation is by vagus nerve (Jamali, Waqar, and Gerson 2016). The diabetic autonomic dysfunction in the early stages affects the longest autonomic nerves, so the vagus nerve (which is the longest nerve in the body and mediates 75% of parasympathetic activity) is affected early. Therefore, there is a decrease in the parasympathetic activity in early stage of DM, which leads to compensatory of sympathetic activity. In addition, progressive DCAN leads to

sympathetic failure in the later stages of DM when the disease becomes clinically evident (Pop-Busui et al. 2004, Taskiran et al. 2004). The clinical manifestation of DCAN in early stages of DM includes exercise intolerance and resting tachycardia due to parasympathetic impairment (Pop-Busui 2012).

DM also impairs the cardiovascular autonomic regulation of the baroreceptor reflex. The arterial baroreceptors are localized in the carotid sinus and aortic arch and are stretch sensitive receptors to physiological stimulation and blood pressure changes. The afferent nerve fibers of the baroreceptor reflex pass through vagus and glossopharyngeal nerve fibers to the central nervous system. The efferent nerve fibers pass via autonomic nerves to the blood vessels and heart. High blood pressure results in an increase of the vagal nerve stimulation and a decrease in sympathetic outflow, resulting in decrease in heart rate (HR), cardiac contractility, and peripheral resistance. On the contrary, low blood pressure leads to inhibition of vagal activity and increase in sympathetic activity causing increase HR, cardiac contractility and peripheral resistance (Kirchheim 1976, La Rovere, Pinna, and Raczak 2008). DM is accompanied by impairment in the baroreceptor reflex that is often associated with life threatening arrhythmia (El-Menyar 2006). The diabetic patients with abnormal baroreceptor reflex have an increased risk for morbidity and mortality than that in diabetic patients with normal baroreceptor reflex (Ewing, Campbell, and Clarke 1980). The diabetic dysfunction in baroreceptor reflex has been demonstrated in the diabetic patients (Kaminska et al. 2008, Martiniskova et al. 2009), and in the experimental diabetic rat model (Dall'Ago et al. 1997, Maeda et al. 1995).

The sympathetic-vagal balance of the autonomic system can be evaluated by several measurements such as heart rate variability (HRV) and baroreceptor reflex sensitivity. There are also other invasive measurement techniques such as microneurography and cardiac sympathetic imaging, but HRV and baroreceptor reflexes assessments are non-invasive techniques that are widely used to evaluate cardiac autonomic innervation (Aubert et al. 1999). Notably, a reduction in HRV is one of the initial signs of cardiovascular autonomic neuropathy (Schonauer et al. 2008). Furthermore, abnormality of HRV in DM represent a higher risk for ventricular dysrhythmia, as well as overall cardiovascular morbidity and mortality

(Bhagyalakshmi et al. 2007). Therefore, measurement of HRV is a simple test for early detection of DCAN, thereby promoting appropriate therapeutic intervention (Boulton et al. 2005).

2.2.3.1 The mechanisms underlying diabetic cardiovascular autonomic neuropathy

There are several mechanisms and pathways involved in the pathogenesis of DCAN. These include metabolic disturbances, neurovascular insufficiency, autoimmunity, and neuro-hormonal disturbances. Hyperglycaemia and its adverse metabolic derangements such as oxidative stress, formation of AGEs mentioned above, all represent important causes for autonomic neuronal damage (Vinik, Freeman, and Erbas 2003). Oxidative stress is a critical pathogenic factor for DCAN. The neuronal axons are more susceptible to oxidative stress damage since the axon has a high content of mitochondria (Leininger et al. 2006, Vincent et al. 2004). Furthermore, in non-diabetic conditions, mitochondrial ATP synthase has been reported to be impaired by oxidative stress damage in the early stages of neurodegenerative disorders (Terni et al. 2010). However, the exact role of mitochondrial oxidative stress on ATP synthase in DCAN is still unknown.

Changes in the neuronal ultrastructure or molecular composition in the heart also contribute in the pathogenesis of DCAN. Sanyal et al. (2012) showed that there was a degradation of parasympathetic nerve endings and a reduction of neurotransmitter vesicles in diabetic rats. In addition, Li et al. (2015) documented that the underlying mechanism of DCAN involves the remodelling of autonomic nerves through derangements in the process of neurotransmitter synthesis. Moreover, Yang and Chon (2011), evaluated autonomic nerve protein markers such as synaptophysin and demonstrated that there was a reduction in the autonomic nerve density in spontaneously diabetic mice.

2.2.4 Electrolyte disturbances in diabetes

DM is often associated with various electrolyte disturbances such as those of potassium, Mg^{2+} , sodium and Ca^{2+} . Electrolytes are an essential for the excitation- and contractile activity of the heart, and for maintenance of cardiac function (Marks

2003). Apart from the aspects of electrical remodelling described above, diabetic electrolyte disturbances have also been reported to underlie myofilament dysfunction in the diabetic heart (Jweied et al. 2005). The dysregulation of ionic channel transport and mitochondrial dysfunction also play a role in the electrolyte disorders in DM (Heyliger, Prakash, and McNeill 1987, Takeda et al. 1996). Among electrolyte disturbances, hypomagnesemia is a common electrolyte disorder in DM with a prevalence rate of about 25-30% (Rude 1992).

2.2.4.1 Mg²⁺ and diabetes

Mg²⁺ plays an essential role as a cofactor for various enzymatic reactions involved in energy metabolism (Laires, Monteiro, and Bicho 2004). In addition, Mg²⁺ is an important factor for various cellular process such as insulin activity, metabolism of glucose, and vascular function (Chetan P. Hans 2002). In non-diabetic conditions, low Mg²⁺ at the cellular level causes an increase production of pro-fibrogenic and pro-inflammatory responses (Kurantsin-Mills et al. 1997, Maier et al. 2004, Shivakumar 2002). In addition, low level of Mg²⁺ is associated with reduction in enzymatic activity involved in antioxidant process such as glutathione reductase (Zhou, Olinescu, and Kummerow 1999).

Hypomagnesemia in DM is associated with atherosclerosis (Atabek et al. 2006), and arrhythmia (Del Gobbo et al. 2012). Hypomagnesemia is also a key factor associated with insulin resistance and vascular diseases in DM (Nadler et al. 1993, Paolisso and Barbagallo 1997). The mechanism underlying Mg²⁺ deficiency in DM is not completely clarified. The transport channels for Mg²⁺ include transient receptor potential (TRP) channels. Among these TRP channels members, the TRP melastatin 6 (TRPM6) is responsible for transcellular Mg²⁺ reabsorption, and is expressed in the kidney and intestine (Voets et al. 2004). Lee and collaborators study (Lee et al. 2006) have shown that the increased Mg²⁺ loss in DM is associated with an increase in the abundance of TRPM6 in the kidney. Another related TRP channel member is TRPM7, which is the most sensitive channel for Mg²⁺ ion and involved in regulation of cellular signalling pathways (Penner and Fleig 2007). TRPM7 channel is highly expressed in the myocardium and it is essential for cardiac automaticity. In addition, the expression of TRPM7 is increases in the hyperglycaemia-induced vascular

endothelial cell injury (Sun et al. 2013). In addition, the osmotic diuresis due to glycosuria in DM may also be linked to Mg^{2+} electrolyte disorders (Liamis et al. 2014).

Mg^{2+} has been shown to activate antioxidant enzymes such as superoxide dismutase, catalase and deficiency of magnesium decrease activation of those enzymes in the heart (Kumar and Shivakumar 1997). Magnesium sulphate ($MgSO_4$) also attenuated oxidative stress by decreasing the production of malondialdehyde in sodium metavanadate-induced lipid peroxidation in non-diabetic rat model (Scibior, Golebiowska, and Niedzwiecka 2013). In addition, magnesium valproate also decreased LV hypertrophy and fibrosis in DM and these effects were mediated by reduction of the oxidative stress biomarkers levels in the heart of diabetic rat model (Patel, Raghunathan, and Porwal 2014).

2.2.5 Problem statement

There is controversy about the role of Mg^{2+} on blood glucose level and hyperlipidaemia in DM as well as on diabetic cardiovascular complications such as cardiac autonomic neuropathy, myocardial fibrosis and ventricular dysfunction. One debatable aspect regards the effect of Mg^{2+} on blood glucose level and hyperlipidaemia in DM. Chronic oral Mg^{2+} supplementation in type 2 diabetic patients has been reported to reduce blood glucose and to modulate the lipid profile (Patel, Raghunathan, and Porwal 2014, Solati et al. 2014, Song et al. 2006). A previous study on diabetic patients with hypomagnesemia found that chronic Mg^{2+} supplementation enhanced insulin sensitivity and metabolic control (Rodriguez-Moran and Guerrero-Romero 2003). The effect of Mg^{2+} on blood glucose may be linked to the circumstance that Mg^{2+} is an important cofactor for glucose transport and it is involved in several enzymatic reactions in carbohydrate metabolism (Laires, Monteiro, and Bicho 2004). However, other studies have found no significant effects of Mg^{2+} supplementation on blood glucose and lipid profile in DM (de Valk et al. 1998, Lal et al. 2003). Although chronic Mg^{2+} administration could modulate lipid profile in type 1 DM (Soltani, Keshavarz, and Dehpour 2007), there is no evidence reported on effect of the Mg^{2+} on blood glucose level in this type of DM.

2.2.6 Significance of the present study

The effects of Mg^{2+} on the diabetic cardiovascular complications have received a growing interest. Previously, Mg^{2+} treatment has been reported to improve hyperglycaemia and dyslipidaemia in DM (Patel, Raghunathan, and Porwal 2014, Solati et al. 2014, Song et al. 2006). Furthermore, a recent study in an acute model of streptozotocin (STZ) induced diabetic rat found that Mg^{2+} treatment prevents the impairment of HRV. In addition, Mg^{2+} also improved left ventricular compliance, but there were no effects of Mg^{2+} administration on blood glucose level, lipid profile or Mg^{2+} level in plasma and tissue (Amoni et al. 2016). However, such effects of Mg^{2+} need to be explored in chronic diabetes to distinguish chronic effects from short term effects. Moreover, there is incomplete evidence about the underlying mechanisms of these Mg^{2+} effects. Therefore, the current study could determine whether these Mg^{2+} effects are sustained in chronic diabetic cardiovascular complications. In addition, this study would help to evaluate the possible underlying mechanisms of Mg^{2+} effects.

Hypothesis

The hypothesis of this study is that the cardiovascular dysfunction caused by chronic DM can be attenuated with Mg^{2+} treatment via mechanisms involving structural- and biochemical alterations.

Aim of the study

To explore the effects of Mg^{2+} treatment on chronic diabetic cardiovascular complications and clarify possible underlying mechanisms.

Specific objectives

- 1- To evaluate the effect of Mg^{2+} treatment on chronic diabetic cardiac autonomic neuropathy, ventricular function and myocardial morphological changes.
- 2- To determine the underlying mechanisms of Mg^{2+} effects.

Chapter 3: Materials and methods

3.1 Ethical statement

The experimental procedures were approved by the Animal Ethics committee of the Faculty of Health Sciences, University of Cape Town (Protocol Ref AEC 014/014, see approval letter in Appendix 1), and were performed in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 2011). The personnel who performed procedures on animal experiments were authorised by the South Africa Veterinary Council.

3.2 Animals and animal care

Adult male Wistar rats (weighting 250-300 g) were used in this study. The Wistar rat strain is the most widely used in cardiovascular research (Berardi et al. 2011, van Dijk et al. 2011). In addition, a previous study in our laboratory successfully established an acute diabetic model using the Wistar strain (Amoni et al. 2016), so for continuity, the same rat strain was used for chronic diabetic model. The reason for the choice of male rats only was to avoid a hormonal cyclic change in the female which could affect the blood glucose level or impair cardiac function (Wong et al. 2013).

The rats were obtained from Stellenbosch University Research Animal Facility and were transferred to the University of Cape Town, Anatomy Building Animal Facility. The rats were given two days to adapt to a new environment before starting the experimental procedures. The rats were housed in clean cages with wood shaving as bedding, and red-rubber tubes were placed in the cages for environmental enrichment. To enhance animal socialization, more than one rat was housed per cage. The environmental temperature was set at 21-23°C and the light intensity at 150 Lux, with fixed 12-12 hours light-dark cycle. The rats had a free access to standard rat chow (Imbani Nutrition, SA), and water. The welfare of the rats was monitored daily.

3.3 Treatment protocol

In this study streptozotocin (STZ; Sigma, SA) was used for induction of DM. STZ (2-Desoxy-2-3-methyl-3-nitrosoureido-D-glucopyranose) is a broad spectrum antibiotic produced by the bacterium *Streptomyces achromogens*, and is particularly toxic to the insulin-producing beta cells of the pancreas through alkylation of the DNA (Elsner et al. 2000). The diabetogenic activity of the STZ was initially described in 1963 (Rakieten, Rakieten, and Nadkarni 1963), and the drug then became a widely used chemical for the experimental diabetic induction in rodents (Lenzen 2008, Szkudelski 2001). STZ produces a diabetic state that mimics type 1 DM that is characterized by hyperglycaemia, polyuria, polydipsia, polyphagia, glycosuria, and hypoinsulinemia (Hakim, Patel, and Goyal 1997). In addition, from the disease model established in our laboratory, STZ diabetic model also has an evidence of hyperlipidaemia, a feature of type 2 DM (Amoni et al. 2016). A moderate dose of the STZ (50 mg/kg body weight) can induce non-ketotic diabetes in rats that can survive for a long duration such as four weeks without a need for insulin treatment (Miethke et al. 1986, Wei et al. 2003). The STZ-induced diabetes is therefore a good model for evaluating the pathogenesis of DM and the mechanism of diabetic complications (Wu and Yan 2015).

The experimental rats were divided randomly into four treatment groups described below (figure 1), and each rat was identified by a special code.

- 1) STZ group: the rats were injected intraperitoneally (i.p) once with STZ (50 mg/kg body weight) thereafter, the rats were injected i.p once daily for 28 consecutive days with normal saline.
- 2) STZ+Mg group: the rats were injected once i.p with STZ (50 mg/kg body weight) thereafter, the rats were injected i.p with MgSO₄ (270 mg/kg body weight) for 28 consecutive days.
- 3) Control group: the rats were injected i.p with STZ vehicle (citrate buffer) once then the rats were injected i.p once daily with normal saline for 28 consecutive days.
- 4) Mg group: the rats were injected i.p once with citrate buffer, thereafter, the rats were injected i.p once daily with MgSO₄ (270 mg /kg body weight) for 28 consecutive days.

STZ was prepared freshly before the time of injection, and was dissolved in 0.1 mol/L citrate buffer at pH 4.5 (see details in Appendix 2). The rats were starved of food for 6 hours before injection in order to increase STZ sensitivity and the uptake of STZ by pancreatic β -cell (Chen et al. 2015). Blood glucose levels were measured using a glucometer (Accu-Chek, Roche, SA) through tail prick blood samples. The rats with random blood glucose levels > 14 mmol/L were considered diabetic. The dose of MgSO_4 (270 mg /kg body weight) was chosen based on a previous study in our laboratory that showed this dose of MgSO_4 had a cardioprotective and antiarrhythmic effects in a cardiac hypertrophy model (Amoni et al. 2017).

The rat body weight and the blood glucose levels were measured daily during the first week of the study, after which the body weight was measured once every three days and the blood glucose was measured once weekly. On day 28, in-vivo HRV parameters were measured using tail pulse plethysmography (n= 9-10 rats/group). Thereafter, ex-vivo experiments were performed for either hemodynamic and electrocardiogram (ECG) assessments on isolated hearts (n= 8-10 rats/groups), or the harvesting of the heart tissue for a histological study (n= 5-6 rats/group) and western blot analysis (n= 3-4 rats/group) as described below.

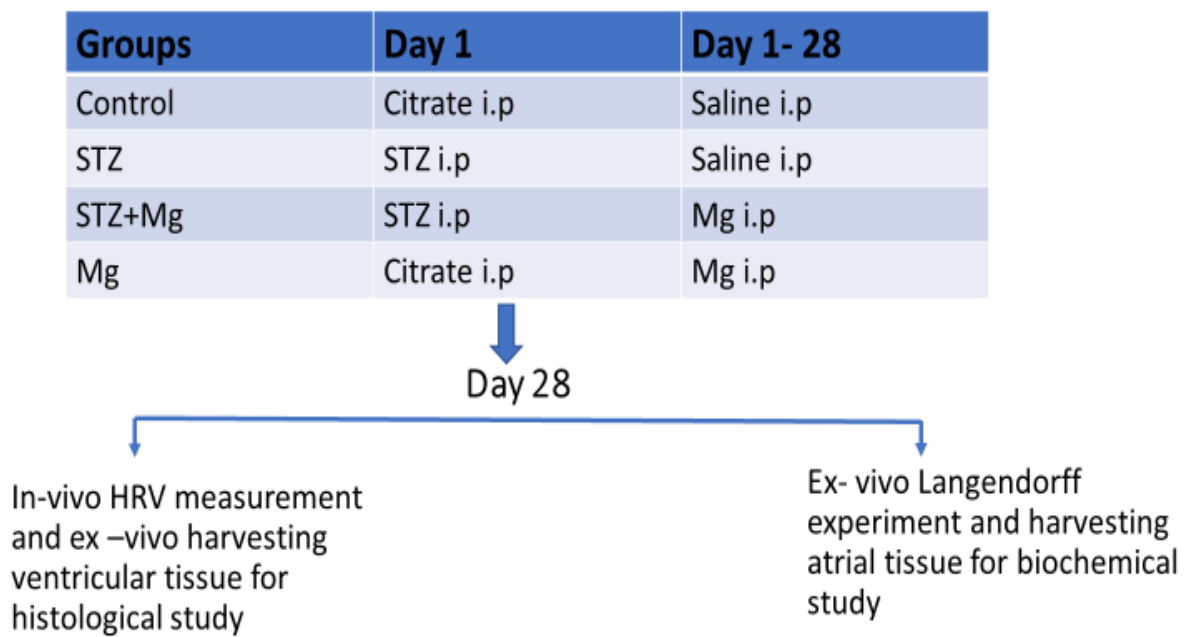


Figure 1: Summary diagram of the experimental protocol.

STZ: streptozotocin, Mg: magnesium, i.p: intraperitoneal.

3.4 In-vivo heart rate variability measurements

The standard technique for HRV measurement is an analysis of the RR intervals from the ECG. However, the use of tail pulse waveform instead of ECG interval for HRV measurement has been validated in other studies, including in our laboratory (Amoni et al. 2016, Linder et al. 2014). The advantages of using tail pulse plethysmography in rats are that the procedure is non-invasive and that there is no need for anaesthesia. Anaesthesia produces sympathetic dysfunction, thereby altering the autonomic function of the heart (Shimokawa et al. 1998).

HRV was measured on day 28 of the experiment using tail pulse plethysmography. The recording was done without anaesthesia, but with a rat held in a restrainer. The measurements were made in a quiet room with regulated temperature (21-23°C) and light intensity in order to avoid stress on the rats. The rats were familiarized with the rodent restrainer daily for one week before actual recordings. The piezo-electric

pulse transducer (TN1012, AD Instruments, Australia) was placed on the base of the rat's tail on the lateral side of the ventral artery to avoid compression (figure 2 A). The transducer was connected to the Power Lab data-acquisition system (AD Instruments, Australia). The pulse recording was done for 5-10 minutes while the rat was in the flat resting position. To induce orthostatic stress, the restrainer was tilted to a 70° angle (figure 2 B), and controlled in this position for recording for an additional 5 minutes. The orthostatic stress increases sympathetic reactivity through activation of the baroreceptor reflex due to changing the position of the body to head-up (Bedette, Santos, and Fontes 2008). HRV parameters were recorded with Power Lab data-acquisition system through LabChart Pro 8 software (AD Instruments, Australia).

The HRV data was analysed with the LabChart Pro HRV v.2.0.1 analysis Module (AD Instruments, Australia). The HRV detector setting was adjusted to allow all beat detection accurately. The recorded trace was scanned manually to exclude all ectopic and artefacts from the analysis. The interpretation of HRV parameters was performed according to guideline of Task Force of The European Society of Cardiology and The North American Society of Pacing and Electrophysiology (1996). The HRV parameters were analysed using time domain and frequency domain parameters. The time domain parameters included the root mean square differences of successive normal-to-normal intervals (RMSSD), whereas the frequency domain parameters included low frequency (LF) power (0.2-0.8 Hz), and high frequency (HF) power (0.8-2.8 Hz).

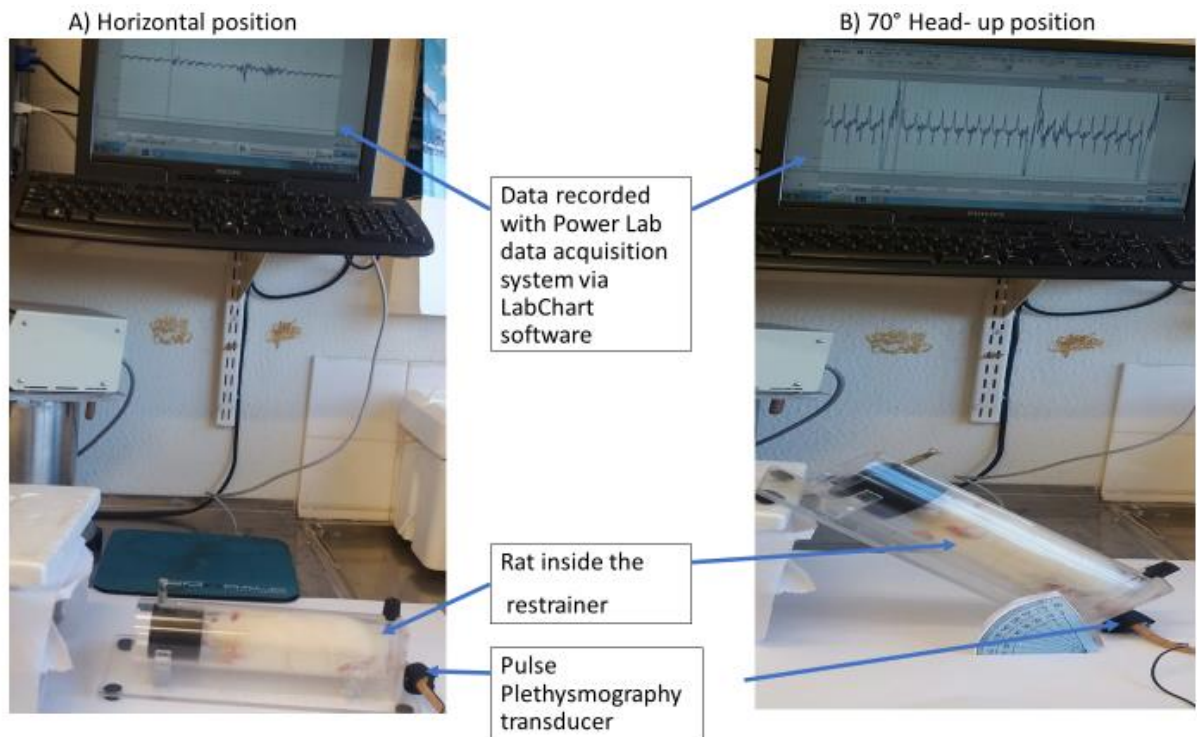


Figure 2: The technique of the HRV recording.

A) Recording in resting position. B) Recording in 70° head -up position. The images were taken from experiments of this study showing the rat inside the restrainer, the tail pulse plethysmography transducer placed at the base of rat tail, and showing how the data were recorded with the Power Lab data acquisition system.

3.5 Heart isolation and perfusion technique

The surgical procedure for heart isolation in rats was performed under general anaesthesia. The rats were anesthetized with sodium pentobarbitone (70 mg/kg body weight, i.p), following the injection with heparin (500 IU /kg body weight, i.p) to prevent thrombus formation. Once the depth of anaesthesia was recognized by the disappearance of the pedal withdrawal reflex, a thoracotomy was performed through an incision from the xiphoid sternum to the left and right costal margin. The anterior chest wall was deflected upward and the pericardium was opened. The heart then was excised at the level of the aorta and quickly transferred to a dish containing cold (4°C) Krebs-Henseleit (K-H) buffer solution that was prepared freshly and contained (in mmol/L) (NaCl 118.5, KCl 4.7, CaCl₂ 1.2, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2 and glucose 11, pH 7.4). The heart was quickly mounted on the Langendorff by aortic cannulation using a stainless-steel cannula and was tied with a 3-0 silk suture

(figure 3). The heart was perfused via retrograde perfusion with the Langendorff perfusion system using K-H buffer bubbled with 95% oxygen and 5% carbon dioxide. The perfusion pressure was kept on a constant pressure (74 mmHg). The temperature of K-H buffer solution was maintained at a constant temperature between 37-38°C by a heated water-jacketed system.

3.5.1 Cardiovascular function assessments

After aortic cannulation, the electrodes of the ECG (an apex to base electrogram that corresponds to the lead II on an ECG) were connected. The ECG electrodes included a positive electrode that was connected around the aorta; a negative electrode was connected to the apex of the heart and a ground electrode that was placed in the free part of the cannula (figure 3). The ECG electrodes were connected to the Power Lab data-acquisition system (AD Instruments, Australia) via an Animal Bio Amplifier (ML136, AD Instruments, Australia).

For cardiac hemodynamic assessments, a deflated balloon was inserted into the LV through the left atrial appendage and the mitral valve. The balloon was connected to a pressure transducer (MLT0699, Australia). The pressure transducer was connected to the Power Lab data-acquisition system (AD Instruments, Australia) through a bridge amplifier (Bridge Amp ML221, AD Instruments, Australia).

Once the Langendorff preparation was established, the balloon was inflated with water to get end diastolic pressure of 5-10 mmHg. Subsequently, the volume of the balloon was not changed, and the heart was perfused without further intervention. The hemodynamic parameters that were measured included the LV developed pressure (LVDP) (difference between systolic and diastolic pressure), HR, the maximal rate of left ventricular pressure increase ($+dP/dt$), and the maximal rate of LV pressure decline ($-dP/dt$). The rate-pressure-product (RPP) was calculated as $LVDP \times HR$. In addition, the coronary flow rate was measured through the collection of the coronary effluent of the hearts over one minute.

The exclusion criteria for all the experiments was as follows: hearts that lasted more than three minutes during aortic cannulation or hearts that failed to beat within sinus rhythm after mounting on the Langendorff perfusion system.

The data was captured and analysed with LabChart v8 software (AD Instruments, Australia). The ECG data was analysed with LabChart Pro ECG analysis v.2.4 module software (AD Instruments, Australia). The haemodynamic data were analysed with LabChart 8 Pro blood pressure module (AD Instruments, Australia).

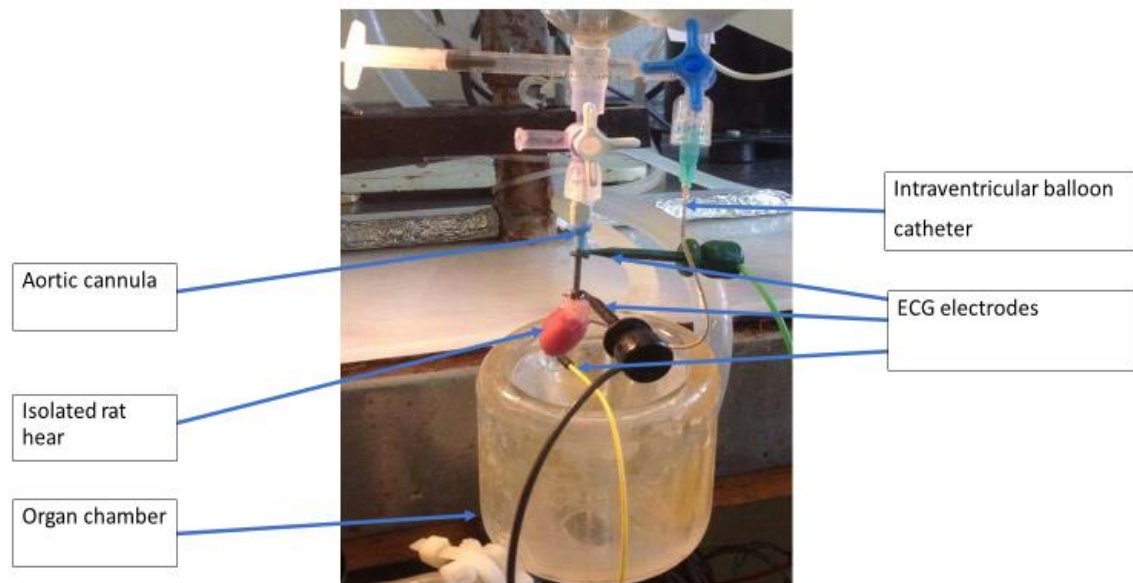


Figure 3: The isolated rat heart mounted on the Langendorff perfusion system. The image shows the isolated rat heart mounted on the Langendorff perfusion system through aortic cannula. The ECG electrodes and the intraventricular balloon are connected.

3.6 Histological studies

Hearts used for histological studies were briefly perfused with K-H buffer solution for 2 minutes to wash out the blood. The hearts were then blotted with tissue paper, weighed, and quickly sliced transversely from the apex into 4 slices (each about 2-mm thickness). The heart slices were then fixed in 10% buffered formalin for 48 hours to prevent degradation of tissue structure and keep integrity of cells and subcellular component. After fixation, the tissue was transferred into 70% alcohol for storage prior to processing.

Tissue processing was performed using an automated tissue processing system (Leica TP1020, Leica Biosystems, Germany), which allowed tissue processing with gentle agitation. The tissue processor system included dehydration in consecutive concentrations of alcohol (70%, 96%, 100%), clearing with xylene and paraffin wax impregnation with molten paraffin wax. Following tissue processing, the tissue was embedded in wax blocks using wax embedding system (WD-4, SA). The embedded tissues were sectioned with a rotary microtome (Leica RM2125RT, Leica Biosystems, Germany) into 4- μ m thick sections. The tissue sections were mounted on glass slides and incubated in hot oven overnight to allow attachment of sections over slides. Tissue sections on slides were stained with Haematoxylin and Eosin (H&E) stain or with Masson's trichrome stain. Detailed staining protocols are described in the Appendix 3.

The slides were digitally scanned with the Olympus VS120 slide scanning system (Olympus VS120-L100 system, Germany). This system allowed for scanning of the whole slide automatically and produced high resolution images. The scanned slides were viewed with Olympus OlyVIA software (OlyVIA 2.8 Viewing software), and the images were captured as screenshots.

The images were digitally analysed with ImageJ software (NHI, USA). Briefly, four images were captured from different areas within each heart and cell widths of 10-12 cells per heart were measured to assess evidence of cardiac hypertrophy. Interstitial fibrosis was semi-quantified by assessing the degree of fibrosis using Masson's trichrome staining as follows: (-); none, (+); mild, (++); moderate, (+++); severe, using guidelines as described by Buwa et al. (2016).

3.7 Western blot analysis

Western blot analysis was used to detect a marker of synaptic degradation synaptophysin, a presynaptic vesicle glycoprotein that is present in neuroendocrine cells and neurons. In addition, western blot was performed to assess a mitochondrial ATP synthase (ATP5A) activity, which is the key component of mitochondrial respiratory function.

The right atrial tissue of the hearts used for western blot studies was separated, snap frozen in liquid nitrogen, and stored at -80°C until used for a western blot analysis. The frozen right atrial tissue was homogenised by sonication (Soniprep 150, UK) for 20 seconds in Radioimmunoprecipitation (RIPA) lysis buffer containing 50 mM Tris-HCl (pH 8), 1% Triton x-100, 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate with a protease and phosphatase cocktail inhibitor (Halt protease and phosphatase inhibitor, ThermoScientific, USA). The lysates were then centrifuged at 15000 relative centrifugal force for 30 minutes at 4°C (Labnet International, NJ07095, USA). The supernatant was collected and the protein concentration of supernatant was measured using the Pierce protein assay kit (Thermo Scientific, Rockford, USA.). The detailed western blot protocol is provided in Appendix 4.

The loading samples were prepared with equal amounts of protein content of the lysate with Lammler dye (Bio-Rad, SA), RIPA buffer and diethylthiostyrazine. The loading samples were then boiled for 4 minutes at 95°C, stored at -20°C and re-boiled for 4 minutes at 95°C, then briefly centrifuged for 30 seconds on the desk mini-centrifuge (Toms, Laboratory Products) before use. The samples were then loaded and electrophoresed on 12% SDS-polyacrylamide gel using Mini-PROTEIN Tetra Cell System (Bio-Rad, SA) at 150 voltage for 90 minutes.

The protein was transferred from the gel to a Polyvinylidene fluoride (PVDF) transfer membrane (Immuno-Blot PVDF Membrane for Protein Blotting, Bio-Rad, SA), using a semi-dry transfer unit (Trans-Blot Turbo Transfer system, Bio-Rad, SA). The transfer process was confirmed by staining the PVDF membrane with ponceau stain. The membranes were then de-stained by washing with distilled water, and were then blocked with 5% non-fat milk in phosphate buffered saline with 0.1% Tween-20 (PBS-T) for 1 hour at room temperature. The membranes were incubated with primary antibodies (mouse monoclonal anti-synaptophysin, ab18008, Abcam, SA) or with Anti-ATP5A antibody (ATP5A: sc-136178 Santa Cruz Biotechnology, USA) against proteins of interest. Both primary antibodies were used at a dilution of 1:5000 in 5% non-fat milk in PBS-T overnight at 4°C. The membranes were then washed with PBS-T and incubated with horseradish peroxidase (HRP), conjugated secondary antibody (Goat Anti-Mouse IgG HRP conjugate, #170-6516, Bio-Rad,

SA), at dilution of 1:10000 in 5% non-fat milk in PBS-T for 2 hours at room temperature. Next, the membranes were washed with PBS-T and then incubated with enhanced chemiluminescence (ECL) detection solution (Clarity Western ECL Substrate, Cat. #170-5060, Bio-Rad, SA) for 1-3 minutes. The membranes were then exposed to x-ray film (Agfar Healthcare, SA) in dark room. The membranes were immersed in a developer and then in a fixative solution for two minutes.

The membranes were then washed with distilled water, stripped with 8% NaOH, and blocked with 5% non-fat milk in PBS-T for 1 hour. The membranes were incubated with housekeeping protein primary antibody (Monoclonal anti-alpha-tubulin antibody produced in mouse, T5168, Sigma-Aldrich, SA) at dilution of 1:3000 in 5% non-fat milk in PBS-T overnight at 4°C. The membranes were then washed with distilled water and incubated with horseradish peroxidase (HRP) conjugated secondary antibody (Goat Anti-Mouse IgG (H+L)-HRP conjugate, #170-6516, Bio-Rad, SA), at dilution of 1:10000 in 5% non-fat milk in PBS-T for 2 hours at room temperature. The membranes were then washed, incubated with ECL, and detected as above.

The x-ray films were scanned and analysed using Image J software (Image J, NIH, USA). The density of each band was expressed as a percentage of the mean density of all the bands. The percentage of each band was then normalised to the percentage density of its respective housekeeping protein bands.

3.8 Plasma Mg²⁺ assay

Blood used for Mg²⁺ assay was collected from the chest cavity immediately after excision of the heart and was centrifuged at 15000 relative centrifugal force (Beckman microfuge, USA) for 15 minutes to obtain plasma. The supernatant plasma was collected and then transferred into well labelled Eppendorf tubes and stored at -80°C for a later biochemical assay. An aliquot 0.5 ml of the plasma was used for measurement of the plasma Mg²⁺ concentration using automated photometric assay (Beckman AU, PathCare, SA).

3.9 Chemicals and reagents

Sodium pentobarbitone was purchased from VetServ Laboratories (Cape Town, SA). All other chemicals were obtained from Sigma (Sigma-Aldrich, SA), unless stated otherwise.

3.10 Statistical analysis

In this study, data were presented as mean \pm S.E.M. Data from multiple groups with normal distribution were compared using one-way analysis of variance (ANOVA) followed by appropriate Tukey's post-hoc test. Successive measurements were compared using repeated measures ANOVA. Comparison of HRV data between the horizontal- and head-up tilt positions were analysed using paired *t*-test. A p-value of < 0.05 was regarded as statistically significant, n: represents the number of animals per group. The statistical analysis was performed by using Statistica 13 and Microcal Origin Lab programmes (Microcal Software, USA).

Chapter 4: Results

4.1 General parameters

4.1.1 Induction of diabetes and survival of the animals

Thirteen out of 16 rats became diabetic with STZ treatment in the STZ group, and 12 out of 13 rats became diabetic in the STZ+Mg treated group. Those rats that did not become diabetic were excluded from the study. In addition, the general animal welfare condition was stable for the period of the study and none of the diabetic or control animals died or were euthanized during the period of the study.

4.1.2 The effect of Mg²⁺ treatment on blood glucose level in STZ-induced diabetic rats

Before STZ injection, there were no significant differences in the blood glucose level in all animal groups (figure 4. A). The non-diabetic control group was euglycemic (range of blood glucose 5-6 mmol/L) over the four weeks of study. STZ treatment induced a significant increase in the blood glucose level from first week onwards, which remained sustained over the four weeks ($P < 0.001$, STZ and STZ+Mg vs. control). The hyperglycaemia in STZ-treated animals was transiently lowered by Mg²⁺ treatment during week 1 and week 3 ($P < 0.01$, STZ+Mg vs. STZ). Mg²⁺ treatment alone did not alter blood glucose level ($P > 0.05$, Mg vs. control).

4.1.3 The effect of Mg²⁺ treatment on the body weight changes in STZ- induced diabetes model

The non-diabetic control animals showed normal body weight gain over the period of the study (figure 4.B). The STZ-treated animals showed loss of body weight from the first week of the study ($P < 0.01$, STZ and STZ+Mg vs. control). Mg²⁺ treatment in STZ-treated animals had no significant effect on the loss of body weight ($P > 0.05$,

STZ+Mg vs. STZ). Mg^{2+} treatment alone did not change the trend of body weight ($P > 0.05$, Mg vs. control).

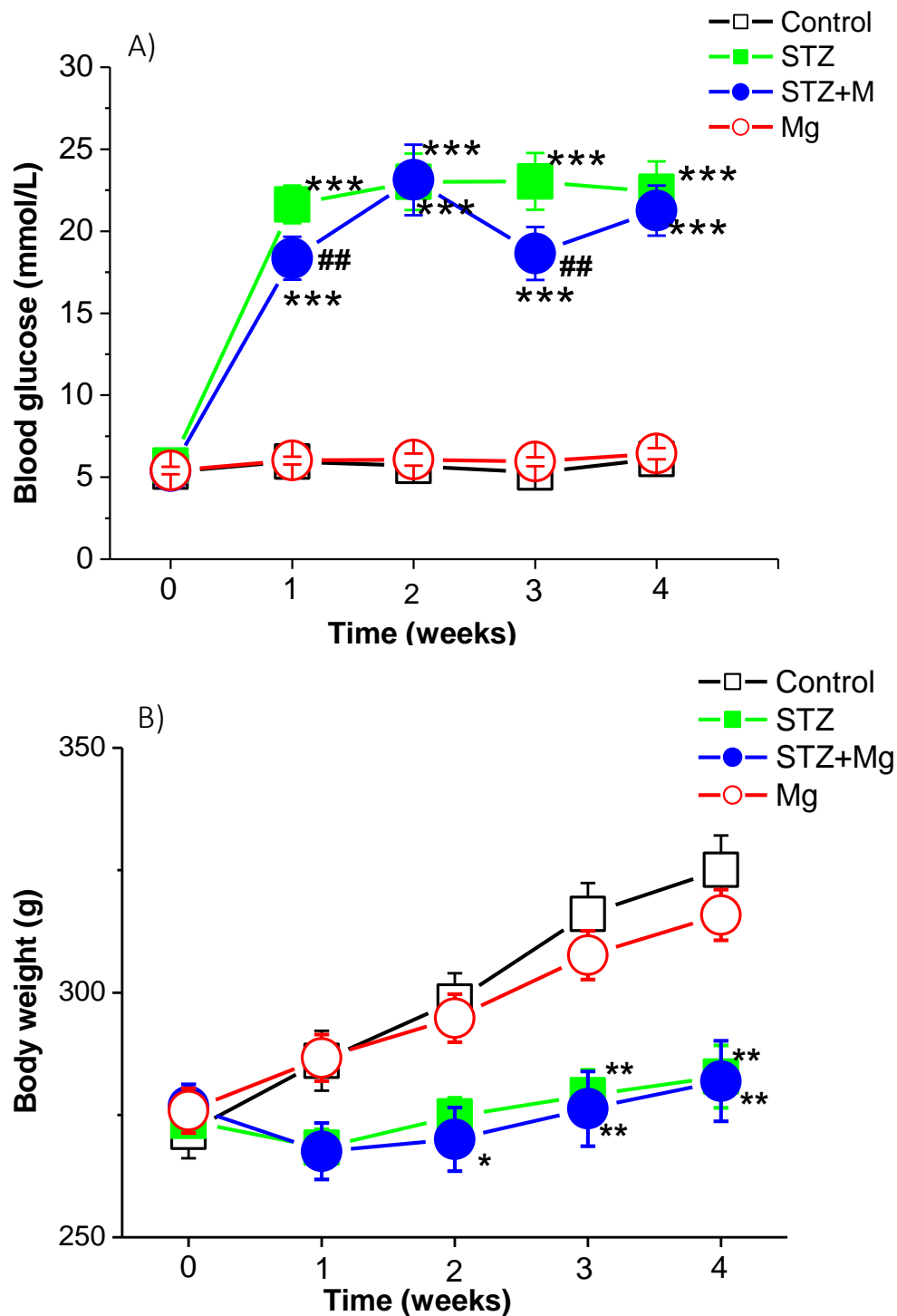


Figure 4: Weekly measurement of blood glucose and body weight for the different treatment groups. A) Weekly blood glucose level for the various treatment groups. B) Weekly body weight changes for all treatment groups. * $P < 0.05$ vs. control; ** $P < 0.01$ vs. control, *** $P < 0.001$ vs. control, and ## $P < 0.01$ vs. STZ, $n = 12-15$.

4.1.4 The effect of Mg^{2+} treatment on the plasma Mg^{2+} concentration in STZ - induced diabetic rats

To find out if the effects of treatment were related to changes in plasma Mg^{2+} level, the plasma concentration of Mg^{2+} at four weeks were analysed. There were no significant differences in the concentration of the plasma Mg^{2+} between the different groups ($P > 0.05$, STZ and STZ+Mg vs. control, figure 5). Mg^{2+} treatment alone had no significant effect on plasma Mg^{2+} concentration ($P > 0.05$, Mg vs. control).

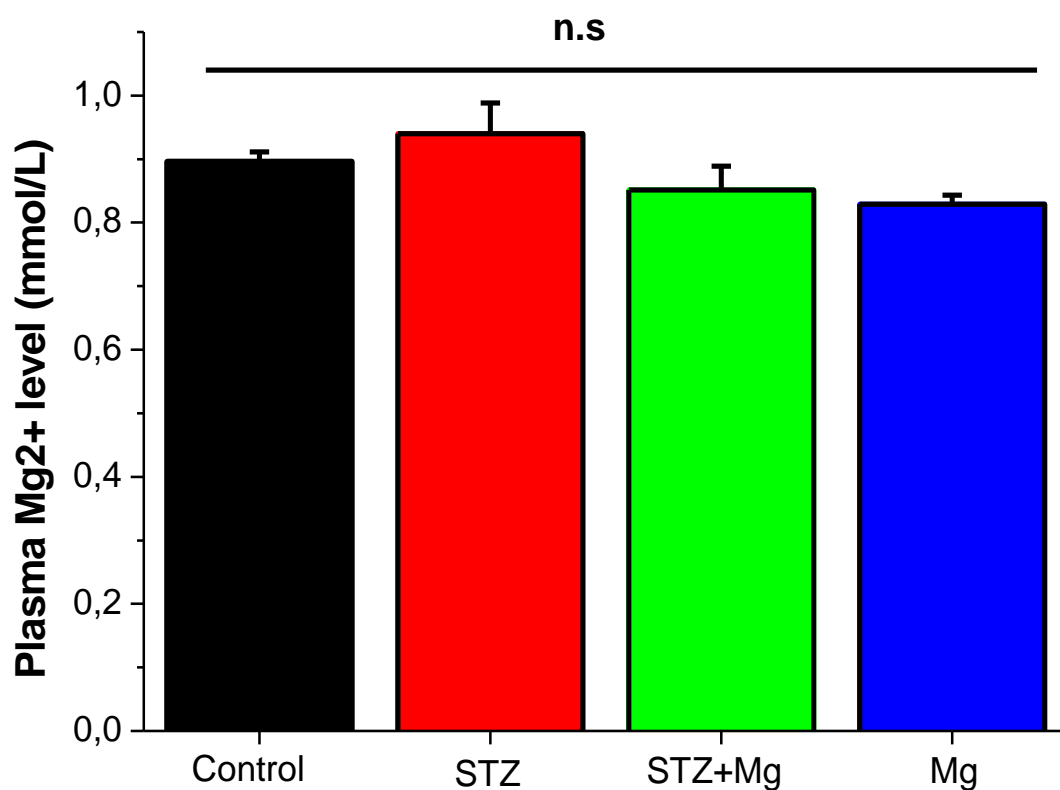


Figure 5: Plasma Mg^{2+} Concentration for the different treatment groups.

Plasma samples were taken at week 4 of the study. n.s: not significant, $n = 8$.

4.2 Magnesium treatment improved heart rate variability in diabetes

4.2.1 The Time Domain parameters of HRV

STZ treatment significantly decreased RMSSD compared to control ($P < 0.05$, STZ vs. control), and this STZ effect was prevented by Mg^{2+} treatment ($P < 0.05$, STZ+Mg vs. STZ) (figure 6. A). Mg^{2+} treatment alone had no significant effect on RMSSD ($P > 0.05$, Mg vs. control).

The non-diabetic control animals had a normal resting HR of about 460 bpm (figure 6. B). STZ significantly reduced HR compared to control group ($P < 0.05$, STZ vs. control), and this STZ effect was reversed by Mg^{2+} treatment ($P < 0.05$, STZ+Mg vs. STZ). Mg^{2+} treatment alone had no significant effect on HR compared to control ($P > 0.05$, Mg vs. control).

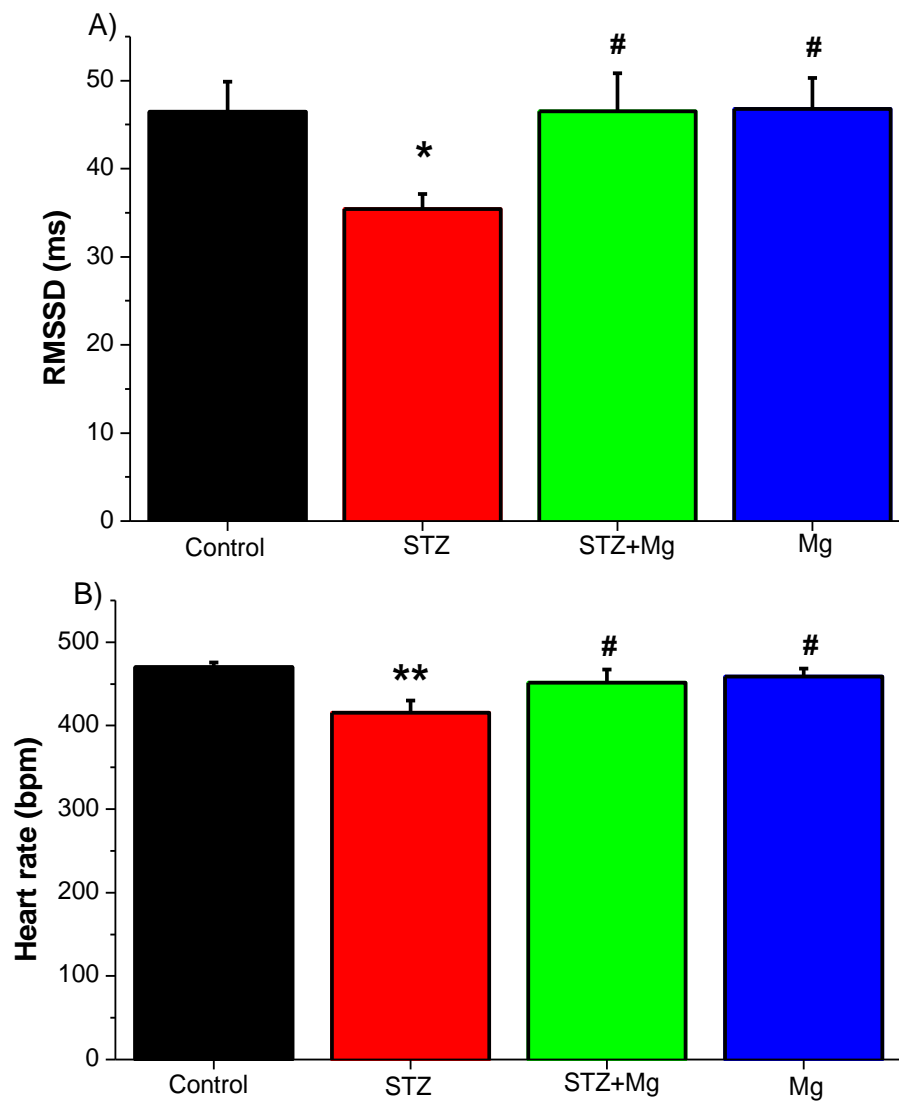


Figure 6: The effects of Mg²⁺ and STZ treatment on the time domain parameter of HRV and HR for the various treatment groups.

A) RMSSD parameter for different treatment groups. B) HR data for various treatment groups. *P < 0.05 vs. control, **P < 0.01 vs. control, and #P < 0.05 vs. STZ. n = 7-9.

4.2.2 The Frequency Domain parameter of HRV

STZ treatment significantly increased LF/HF ratio compared to control group ($P < 0.01$, STZ vs. control, figure 7). This effect of STZ on LF/HF ratio was prevented by Mg^{2+} treatment ($P < 0.01$, STZ+Mg vs. STZ). Mg^{2+} treatment alone had no significant effect on LF/HF ratio compared to control ($P > 0.05$, Mg vs. control).

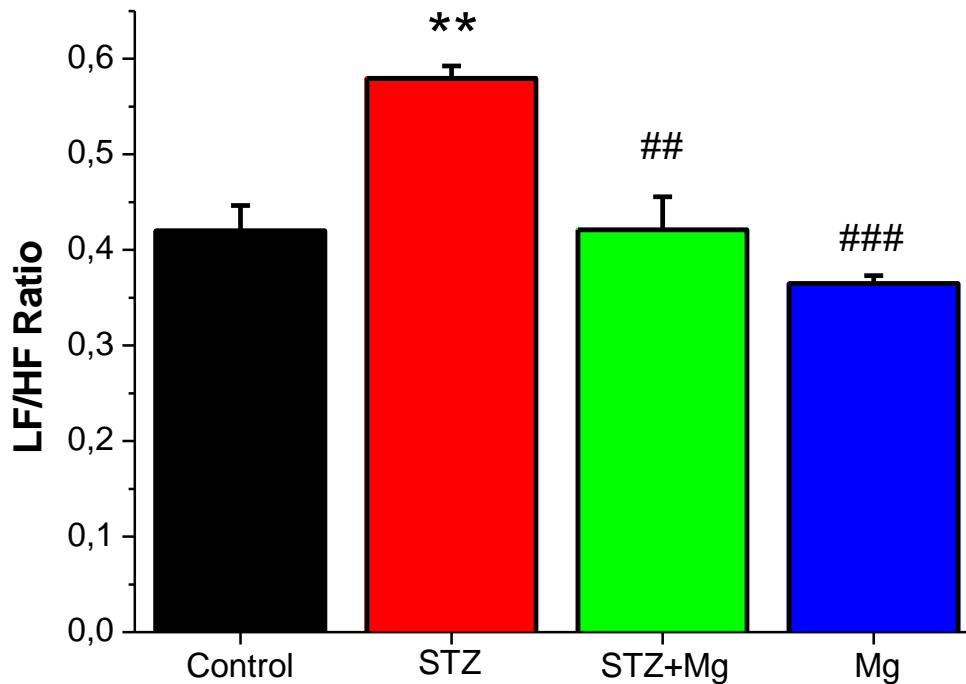


Figure 7: The effects of Mg^{2+} and STZ treatment on frequency domain parameter for the different treatment groups.

** $P < 0.01$ vs. control, ## $P < 0.01$, and ### $P < 0.001$ vs. STZ, $n = 7-9$.

4.2.3 The orthostatic stress response of heart rate

The orthostatic stress test was performed to explore the changes of HR from resting horizontal position to 70° head-up position. The non-diabetic control animals showed normal response orthostatic stress induced tachycardia in the tilt position compared to horizontal resting position ($P < 0.05$, control 70° position vs. horizontal position, figure 8). The STZ-treated animals showed impaired HR response to orthostatic stress compared to resting position ($P > 0.05$, STZ 70° position vs. horizontal position). This STZ effect was improved by Mg^{2+} treatment ($P < 0.05$, STZ+Mg 70°

position vs. horizontal position). The Mg^{2+} treatment alone showed normal response orthostatic stress induced tachycardia ($P < 0.05$, Mg 70° position vs. horizontal position).

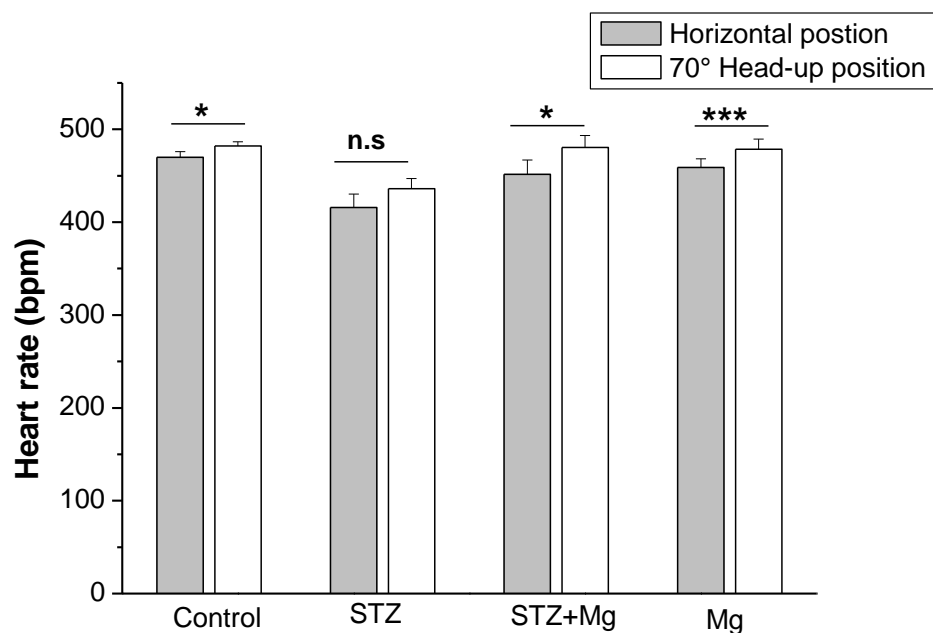


Figure 8: The effects of STZ and Mg^{2+} treatment on the response of HR to orthostatic stress test.

* $P < 0.05$, *** $P < 0.001$: head-up position vs. horizontal position. n.s: not significant, $n = 7-9$.

4.3 The effects of Mg^{2+} and STZ treatment on cardiac synaptophysin protein expression

To evaluate the mechanism through which the Mg^{2+} effects on autonomic dysfunction may occur, western blot analysis of autonomic synaptic innervation was performed. Representative image of western blot analysis in Figure 9 shows the bands of proteins in different treatment groups for synaptophysin and a housekeeping protein (alpha tubulin). Generally, there was an absence of synaptophysin protein bands in three among of four western blot analysis in STZ only treated group, but the protein was present consistently in the other groups.

Qualitatively, western blot analysis showed a decrease in the expression of synaptophysin protein in STZ-treated only group (STZ) compared to control groups. In addition, qualitatively Mg^{2+} treatment improved synaptophysin protein expression in STZ group (STZ+Mg). Mg^{2+} treatment alone showed similar pattern of synaptophysin expression similar to the control group.

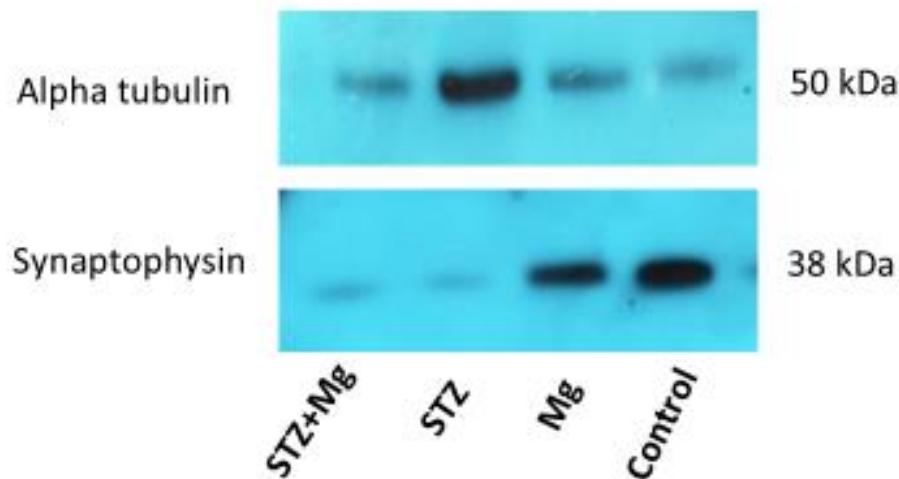


Figure 9: Representative immunoblot bands of synaptophysin and alpha tubulin proteins for the various treatment groups. n= 3-4.

4.4 The effects of Mg^{2+} and STZ treatment on cardiac mitochondrial ATP5A expression

To explore the role of mitochondrial oxidative stress in cardiac autonomic dysfunction, western blot analysis was performed for mitochondrial ATP synthase (ATP5A) expression in the heart. Representative image of western blot analysis (figure 10) showed the bands of ATP5A and alpha tubulin proteins for all treatment groups. Qualitatively, western blot analysis showed the expression of mitochondrial ATP5A among various treatment groups.

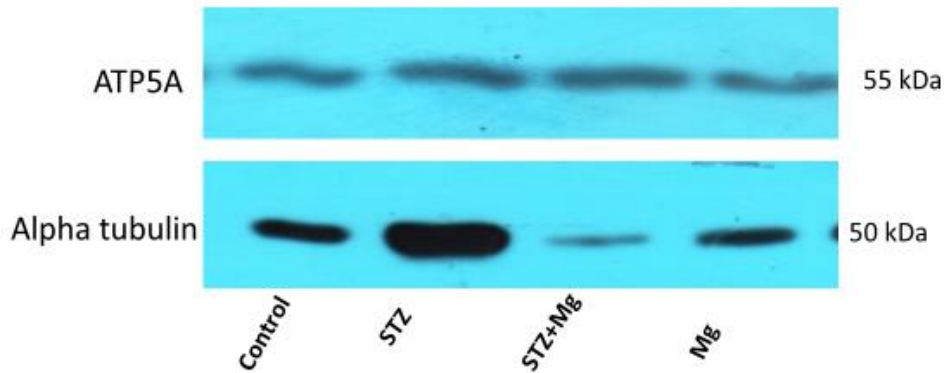


Figure 10: Representative immunoblot bands for ATP5A and alpha tubulin proteins for the different treatment groups. n= 3-4.

4.5 The effects of Mg²⁺ treatment on ECG changes in STZ-induced diabetic model

The representative traces of ECG waveform are shown in figure 11, and the changes in ECG parameters for various treatment groups are summarized in table 1. The STZ treatment significantly decreased heart rate ($P < 0.01$, STZ vs. control). Mg²⁺ treatment did not affect STZ-induced bradycardia ($P > 0.05$, STZ+Mg vs. STZ or control), and had no significant effect on RR interval ($P > 0.05$, STZ+Mg vs. STZ or control). Mg²⁺ treatment alone had no significant effects on HR ($P > 0.05$, Mg vs. control), or RR interval ($P > 0.05$, Mg vs. control). There were no significant differences of R wave amplitude, S wave amplitude, T wave amplitude, QRS interval, QT interval, and corrected QT interval (QTc) among all groups.

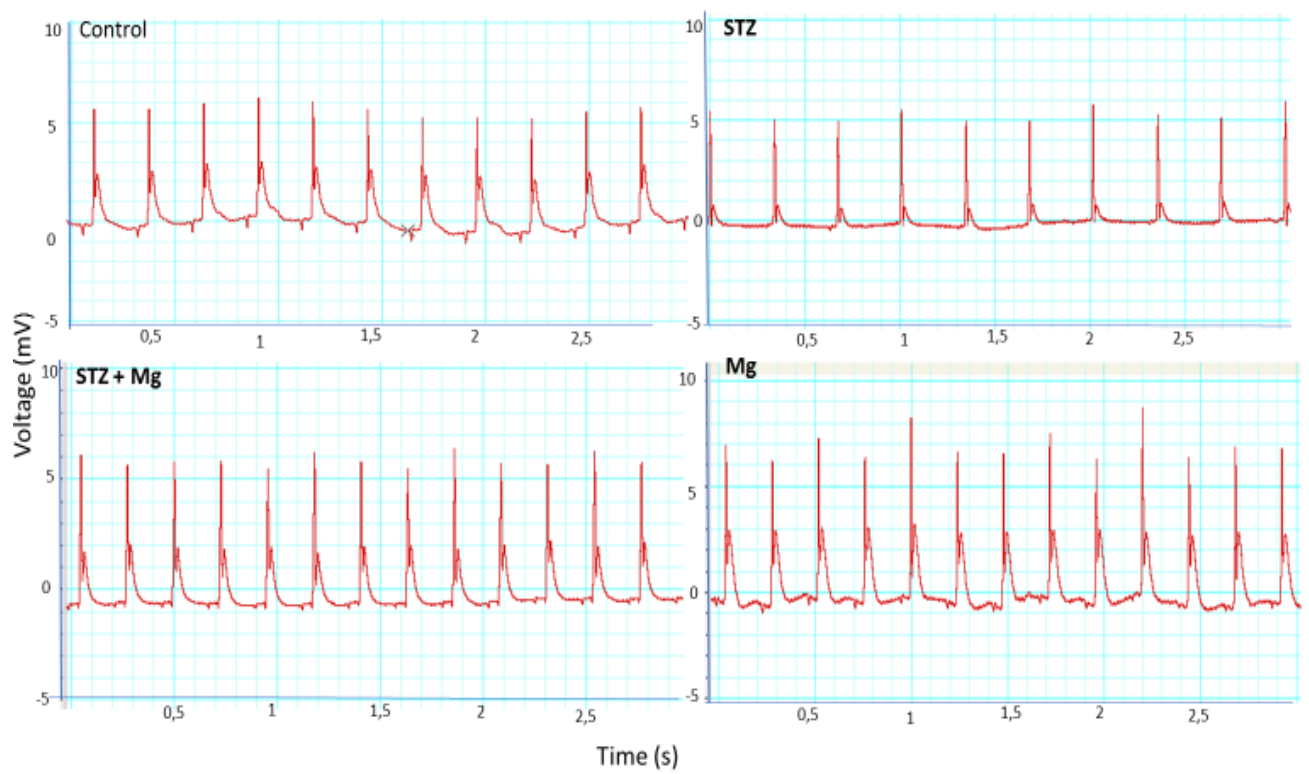


Figure 11: The effects of Mg^{2+} and STZ treatment on ECG waveforms. Representative images of ECG waveforms for the different treatment groups.

Table 1: Summary data of ECG characteristics for various treatment groups.

ECG parameter	Control	STZ	STZ+Mg	Mg
Heart rate (bpm)	233 ± 8	178 ± 14*	209 ± 11	234 ± 13
RR interval (s)	0.27 ± 0.01	0.34 ± 0.03*	0.29 ± 0.02	0.26 ± 0.01
R amplitude (mV)	5.2 ± 0.7	5.6 ± 1.3	6.2 ± 1.1	6.2 ± 0.8
S amplitude (mV)	1.75 ± 0.27	2.1 ± 0.63	2.35 ± 0.73	0.40 ± 1.38
T amplitude (mV)	2.12 ± 0.53	2.56 ± 0.67	2.73 ± 0.95	1.76 ± 0.46
QRS interval (s)	0.020 ± 0.003	0.024 ± 0.002	0.026 ± 0.006	0.024 ± 0.003
QT interval (s)	0.062 ± 0.002	0.079 ± 0.009*	0.065 ± 0.005	0.064 ± 0.006
QTc (s)	0.124 ± 0.006	0.137 ± 0.016	0.119 ± 0.007	0.121 ± 0.009

QTc was calculated with Bazett's formula. Data are presented as mean ± SEM, *P < 0.05 vs. control. n= 7 -11.

4.6 The effects of Mg²⁺ treatment on haemodynamic parameters in STZ-induced diabetic rat model

4.6.1 Mg²⁺ treatment improved hemodynamic function

Generally, the non-diabetic control animals had LVDP of about 100 mmHg. The STZ-treated animals showed a significant decreased LVDP compared to control animals (P < 0.05, STZ vs. control, figure 12 A). Mg²⁺ treatment significantly prevented STZ-induced decreased LVDP (P < 0.05, STZ+Mg vs. STZ). Mg²⁺ treatment alone had no significant effect on LVDP (P > 0.05, Mg vs. control).

The STZ-treated animals also showed a reduction in the maximal rate of LV pressure increase (+dP/dt) compared to the control animals (P < 0.01, STZ vs. control, figure 12 B). This STZ effect on +dP/dt reversed by Mg²⁺ treatment (P < 0.05, STZ+Mg vs. STZ). The STZ-treated animals showed a decrease in the maximal rate of LV pressure decline (-dP/dt) compared to the control animals (P < 0.05, STZ vs. control, figure 12 C), which was not prevented by Mg²⁺ treatment (P > 0.05, STZ+Mg vs. STZ or control). Mg²⁺ treatment alone had no significant effects on +dP/dt and -dP/dt (P > 0.05, Mg vs. control). There were no significant differences

between the different treatment groups in RPP, systolic duration, and diastolic duration (figure 12 D-F).

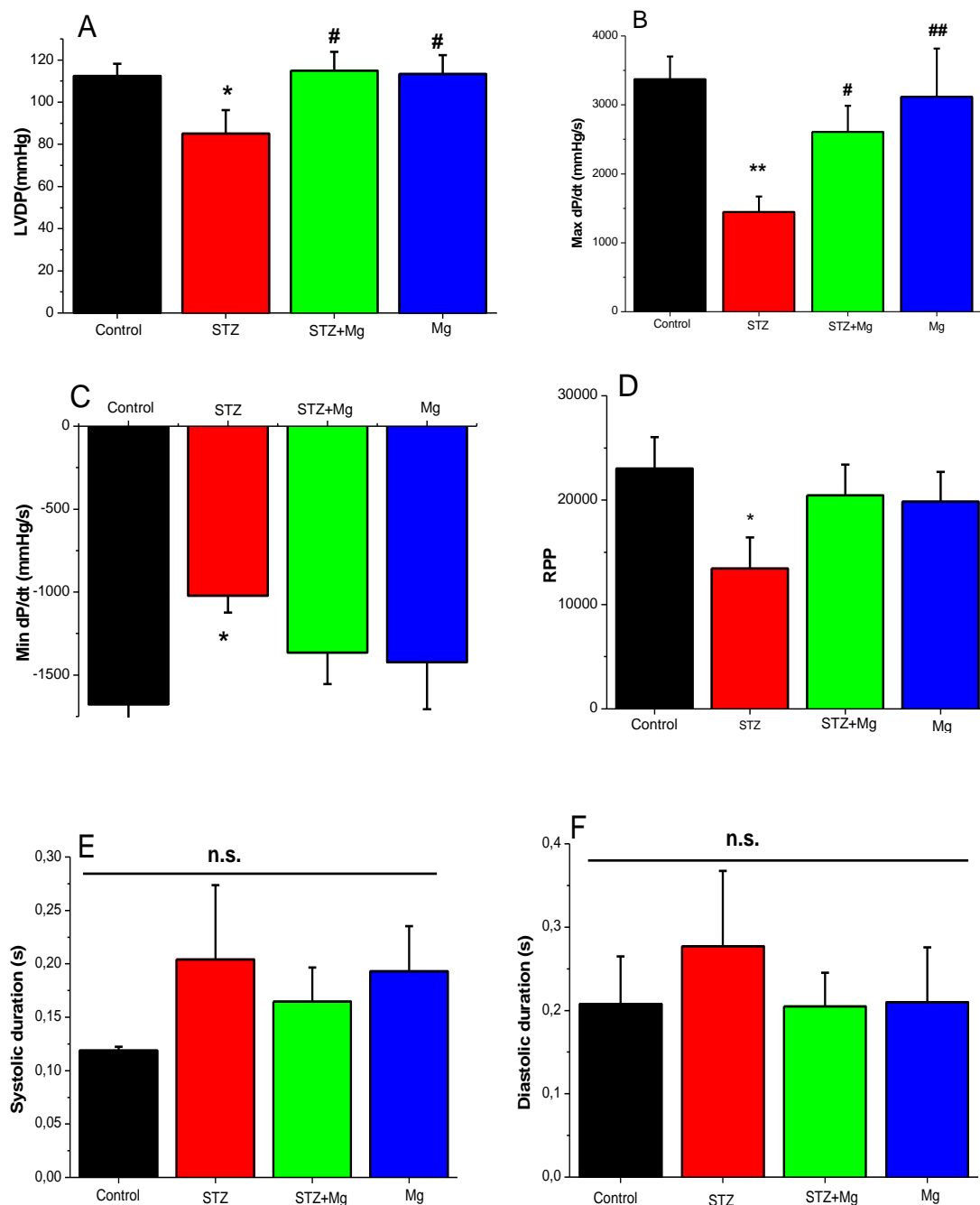


Figure 12: The effects of STZ and Mg^{2+} treatment on haemodynamic parameters for various treatment groups. The haemodynamic parameters includes A: left ventricular developed pressure (LVDP), B: maximal rate of LV pressure increase (+dp/dt), C: maximal rate of LV pressure decline (- dp/dt), D: rate pressure product, E: systolic duration and F: diastolic duration. *P < 0.05 vs. control, **P < 0.01 vs. control, #P < 0.05 vs. STZ, and ##P < 0.01 vs. STZ, n.s: not significant, n = 5-8.

4.6.2 The effects of STZ and Mg²⁺ treatment on coronary flow rate

To further explore the mechanisms underlying the improvement of contractile function by Mg²⁺ in diabetes, the changes in the coronary flow rate were analysed. There were no significant differences between different treatment groups in coronary flow rate (figure 13) ($P > 0.05$, STZ and STZ +Mg vs. control or Mg).

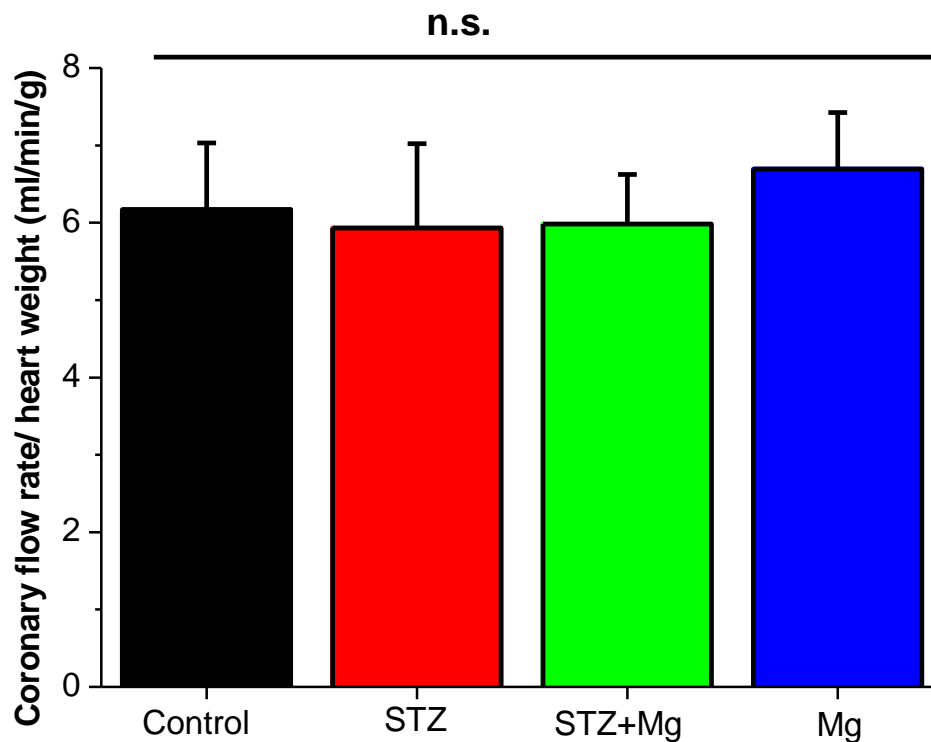


Figure 13: The effects of STZ and Mg²⁺ treatment on coronary flow rate for different treatment groups.

The coronary flow rate was normalized to heart weight. n.s: not significant, n= 7-9.

4.7 The effects of STZ and Mg²⁺ treatment on cardiac structure

4.7.1 Changes in heart weight

The hearts were weighed to assess if there were changes in gross cardiac morphology. The heart weight was normalized to the body weight (body weight on

day 28 of the study) (HW/BW ratio). There were no significant differences in HW/BW ratio among the different groups ($P > 0.05$, STZ and STZ+Mg vs. control or Mg) (figure 14).

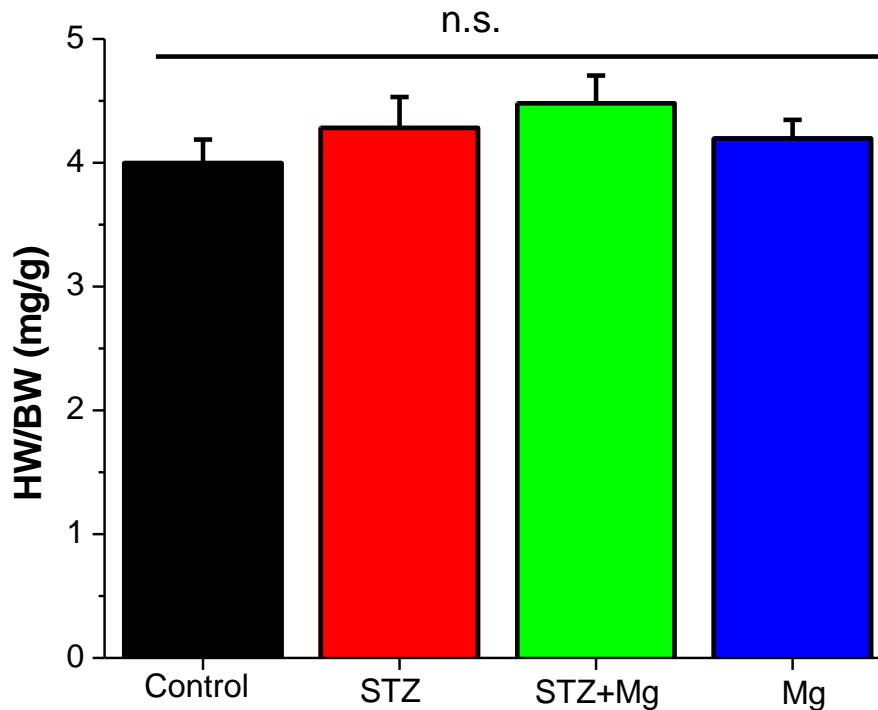


Figure 14: The effects of STZ and Mg^{2+} treatment on heart weight/ body weight ratio. n.s: not significant, $n = 7-9$.

4.7.2 Analysis of cardiac cell size

Cardiac histological features were also analysed in order to infer cardiac function improvements in diabetes. Representative images of H&E stained ventricular tissue for various treatment groups (figure 15 A). The images showed regularly arranged cardiac myofibers and uniform sizes of spaces between cells that are consistent with lack of interstitial infiltrations. Cardiomyocytes widths were measured as an index of cardiomyocyte hypertrophy. There were no significant differences in cardiomyocytes widths among the groups ($P > 0.05$, for STZ and STZ+Mg vs. control or Mg) (figure 15 B).

A

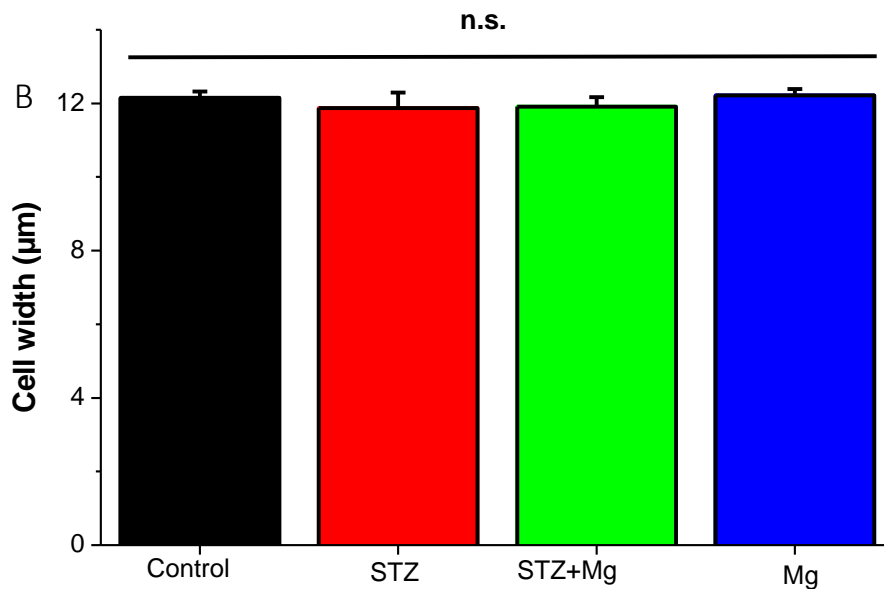
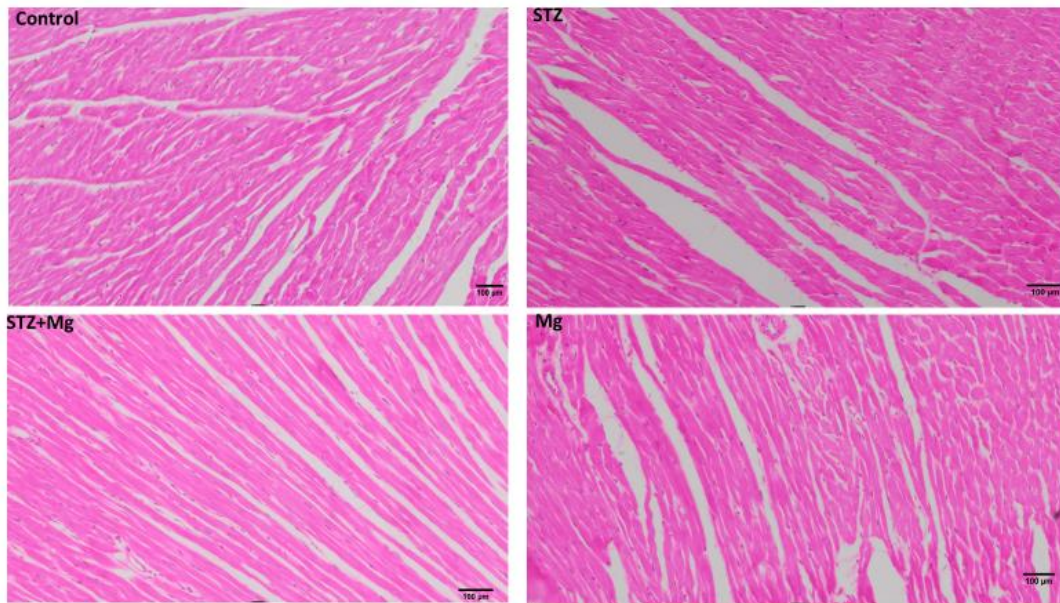


Figure 15: The effects of STZ and Mg^{2+} treatment on histology of cardiomyocyte.

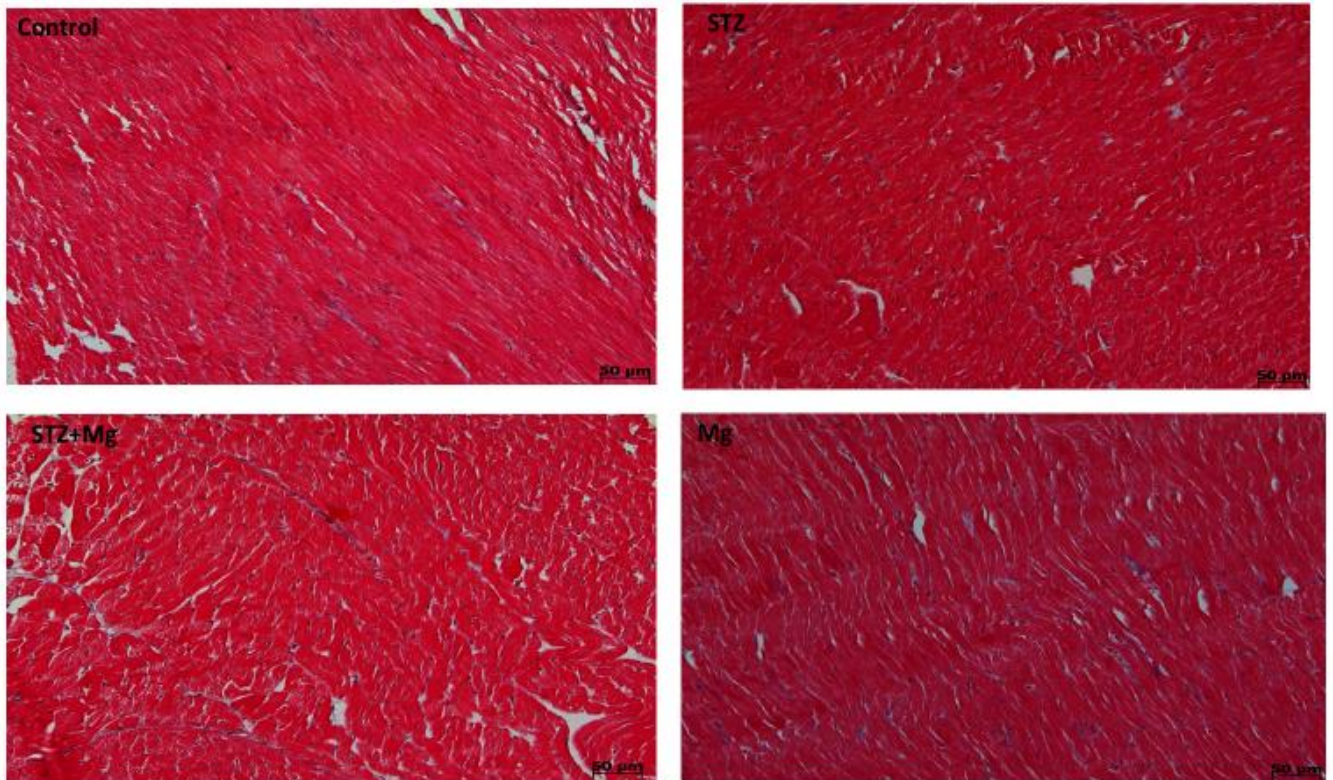
A) Representative images of histological staining (H&E) of ventricular tissue, scale bar in lower right corner represent 100 μm . B) Cardiac cell width analysis for various groups. n.s: not significant, n= 5-6.

4.7.3 Analysis of ventricular tissue fibrosis

Examples of images of Masson's trichrome stained ventricular tissue showed no interstitial fibrosis seen in all groups (figure 16). Furthermore, representative images

of Masson's trichrome stained perivascular tissue (figure 17) also showed no detectable perivascular fibrosis among the different treatment groups.

A)



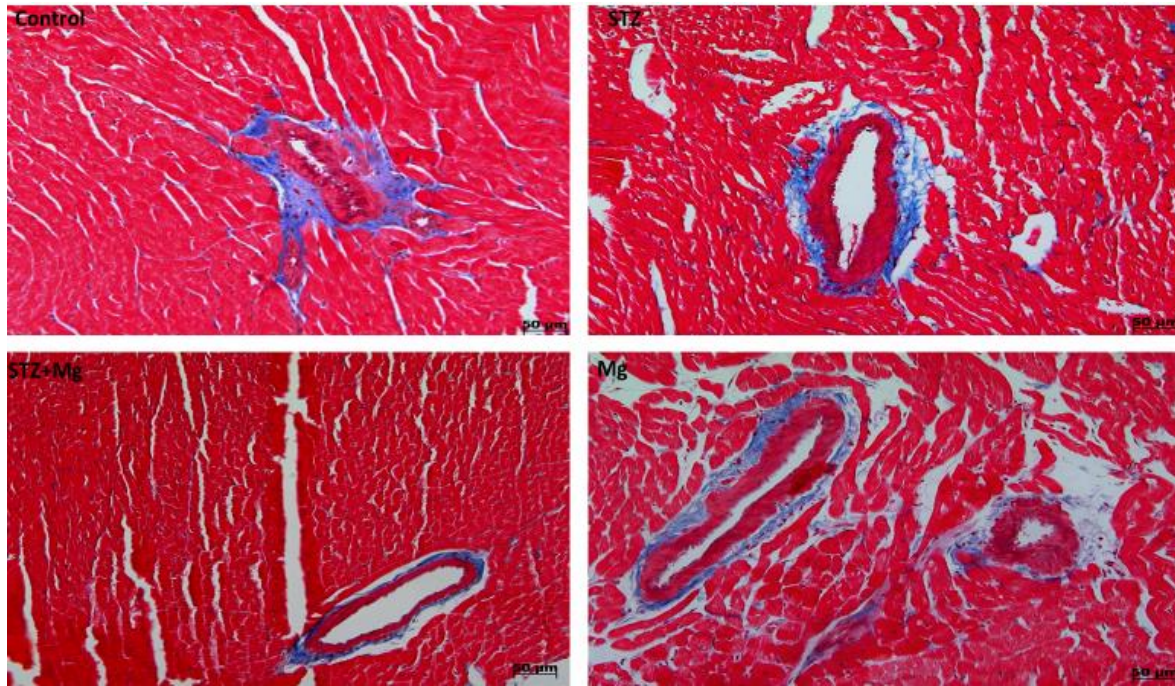
B)

Groups	Interstitial fibrosis
Control	–
STZ	–
STZ+Mg	–
Mg	–

Figure 16: The effects of Mg^{2+} and STZ treatment on the extent of interstitial fibrosis for different treatment groups.

Representative images of Masson's trichrome stained ventricular tissue showed: A) interstitial area. The labelled scale bar in the bottom represents 50 μm . B) Arbitrary score of the extent of interstitial fibrosis for the different treatment groups. (-) depicts none, n = 5-6.

A)



B)

Groups	Perivascular fibrosis
Control	—
STZ	—
STZ+Mg	—
Mg	—

Figure 17: The effects of STZ and Mg^{2+} treatment on the extent of perivascular fibrosis for the various treatment groups.

Representative images of Masson's trichrome stained ventricular tissue showed: A) perivascular area. The labelled scale bar in the bottom represents 50 μm . B) Arbitrary score of the extent of perivascular fibrosis for the different treatment groups. (-) depicts none, n = 5-6.

Chapter 5: Discussion

In the current study, we have shown that the chronic DM is associated with cardiovascular complications that include cardiac autonomic dysfunction and impairment in cardiac hemodynamic function. Daily Mg^{2+} treatment in chronic diabetes attenuated cardiac ventricular dysfunction without affecting coronary flow rate or ECG parameters. Mg^{2+} administration also improved HRV impairment in chronic DM, without evidence of mitochondrial oxidative marker of ATP5A. However, neither the blood glucose level nor plasma Mg^{2+} concentration or cardiac morphological structures were affected by Mg^{2+} treatment.

Diabetic animals showed hyperglycaemia throughout the four weeks. Treatment with Mg^{2+} did not affect the induction rate of diabetes or the hyperglycaemia. This result was supported by previous studies, which showed that the administration of Mg^{2+} does not improve hyperglycaemia or the diabetic induction in both chronic diabetic animal model (Hans, Chaudhary, and Bansal 2003, Rondon et al. 2010) and in diabetic patients (de Valk et al. 1998). In contrast to our results, some studies showed that the Mg^{2+} treatment improved hyperglycaemia in diabetic model (Hasanein et al. 2006, Parvizi et al. 2014, Patel, Raghunathan, and Porwal 2014, Soltani et al. 2005). Soltani et al. (2005) showed histological evidence that Mg^{2+} administration has a protective effect on the pancreatic β -cell from the cell destruction induced by STZ.

In the present study, DM-induced decreased RMSSD, which is indicative of parasympathetic activity according to Task Force of The European Society of Cardiology and The North American Society of Pacing and Electrophysiology (1996). DM also induced increased LF/HF ratio, which is a marker of enhanced sympathetic activity. Therefore, the present study based on these HRV parameters showed that there was an impairment of both parasympathetic and sympathetic activity in chronic diabetes. These results are consistent with previous studies where there was an impairment of both sympathetic and parasympathetic cardiac autonomic function in chronic STZ-induced diabetic rat model (Li et al. 2015, Sanyal et al. 2012). Sanyal et al. (2012) showed that the parasympathetic autonomic impairment occurred in early

onset of DM, while the sympathetic autonomic disturbances happened much later after the onset of DM.

In the current study, DM also impaired orthostatic stress-induced tachycardia. The orthostatic response is indicative of the integrity of baroreceptor reflex (Cowley, Liard, and Guyton 1973, Steinback et al. 2005). The impairment of baroreceptor reflex has been observed in short term STZ-induced diabetes (Amoni et al. 2016, Dall'Ago et al. 2002, Maeda et al. 1995). Therefore, taken together, the impairment in HRV parameters and orthostatic stress response in the present study indicate that the DM-induced cardiac autonomic dysfunction.

In the present study, MgSO₄ administration improved diabetes-induced decreased RMSSD, increased LF/HF ratio, and impaired orthostatic stress response in chronic diabetic model. Therefore, the findings of this study suggested that the Mg²⁺ administration could prevent diabetes induced HRV impairment in chronic diabetes. Several studies have also reported that the Mg²⁺ treatment has a beneficial effect in neuronal studies. For instance, Sameshima, Ota, and Ikenoue (1999) showed that the Mg²⁺ treatment protects the brain against ischemic injury in neonatal rats. In addition, Begon et al. (2000) demonstrated that the Mg²⁺ treatment in STZ-induced diabetic rats improves neuropathic pain. In cardiovascular conditions, Mg²⁺ administration improved HRV impairment in non-diabetic patients with systolic heart failure (Almoznino-Sarafian et al. 2009). Furthermore, in our laboratory study by Amoni et al. (2016) showed that the Mg²⁺ treatment improved HRV disturbances in acute STZ-induced diabetes in rats. Therefore, the findings of the current study are novel in chronic diabetes and suggest that the effect of Mg²⁺ is long lasting.

Based on the observation of synaptic innervation of the present study, the possible underlying mechanism of our results is that the Mg²⁺ treatment may involve the preservation of synaptophysin innervation in the diabetic animals. This mechanism for Mg²⁺ effect is consistent with a study by Sanyal et al. (2012) where there was degradation in the synaptic autonomic innervation in chronic STZ-induced diabetic rat. Their findings were observed through ultrastructural studies of sinoatrial region. Additionally, Yang and Chon (2011) demonstrated that there was a reduction of nerve density (as was evidenced by the decreased expression of synaptophysin) in spontaneously diabetic mice.

Furthermore, Mg^{2+} effects were unrelated to its effect on the expression of mitochondrial ATP synthase in the right atrium, since there was no apparent change in the expression of the protein. However, this finding does not exclude oxidative stress as an underlying mechanism for cardiac autonomic dysfunction because the other important markers of oxidative stress in DM such as malondialdehyde and superoxide dismutase were not evaluated in this study. These oxidative stress parameters have been implicated in STZ-induced diabetic rats, and were prevented by Mg^{2+} treatment (Patel, Raghunathan, and Porwal 2014).

The diabetic animals in this study also showed baseline bradycardia, which is inconsistent with impaired parasympathetic activity suggested by the HRV findings. Similar findings were observed in previous studies where diabetes-induced resting bradycardia in animal disease models (Borges et al. 2006, Dowell, Atkins, and Love 1986, Jackson and Carrier 1983). In addition, in our study bradycardia was also seen in ex-vivo Langendorff perfused experiments, and this finding is also consistent with previous studies (Howarth et al. 2007, Howarth and Qureshi 2006). Senges et al. (1980) have suggested that the bradycardia in diabetic model is due to derangement in an intrinsic electrophysiological activity of sinoatrial node.

Our study also showed decreased LVDP, RPP, $+dP/dt$, and $-dP/dt$ in diabetes, all of which suggest that there was impaired LV contractile function in chronic diabetes. The $+dP/dt$ has been used generally as an index of cardiac contractility, while the $-dP/dt$ is an indicative of diastolic relaxation (Kass et al. 1987, Schmidt and Hoppe 1978). The RPP is a reliable predictor of myocardial oxygen demand (Forjaz et al. 1998, May and Nagle 1984). The results of this study are consistent with those reported in previous studies (Litwin et al. 1990, Radovits et al. 2009) where there was a decrease in the LV systolic pressure, $+dP/dt$ and $-dP/dt$. Litwin et al. (1990) found that the cardiac dysfunction could be reversed by insulin administration. In addition, impaired cardiac performance has been documented in isolated cardiac cells (Ren and Bode 2000). Moreover, several studies showed that there were disturbances in cardiac contractility in diabetic disease models (Hamblin et al. 2007, Trost et al. 2002). In contrast to the findings in our study and from several other studies, Connelly et al. (2007) have reported that there was unaltered cardiac function in chronic STZ-induced diabetic rats. However, their diabetic rats were treated with insulin, which could have masked the incidence of diabetic cardiac

dysfunction. Insulin treatment also increases myocardium Ca^{2+} uptake, therefore it is an important factor for cardiac function (Gotzsche 1985).

Mg^{2+} administration in this study prevented chronic diabetes-induced changes in LVDP and $+\text{dP}/\text{dt}$, but did not affect $-\text{dP}/\text{dt}$ or RPP. Our findings are in agreement with those of previous studies that reported that the Mg^{2+} has a cardioprotective effect in DM. In our laboratory, Amoni et al. (2016) demonstrated a similar effect of Mg^{2+} in preventing ventricular dysfunction in acute STZ-induced diabetic rat. Another study by Amoni et al. (2017) also demonstrated cardioprotective effects of Mg^{2+} in non-diabetic cardiac hypertrophic rat model. In addition, the study by Patel, Raghunathan, and Porwal (2014) reported that the Mg^{2+} valproate supplementation improved hemodynamic dysfunction in chronic STZ-induced diabetic rats. The beneficial effect of Mg^{2+} valproate was proposed to be underlined by the attenuation of oxidative stress in DM. Nevertheless, it is not clear whether this effect is related to Mg^{2+} ion or to the valproate component. Furthermore, in non-diabetic conditions Mg^{2+} administration prevented ventricular dysfunction induced by ritonavir through improvement of oxidative stress and hyperlipidaemia disorders in the rat model (Mak et al. 2013). Therefore, the result of the current study suggests that Mg^{2+} treatment could improve cardiac contractile dysfunction in chronic diabetes.

The mechanisms underlying the improvements in ventricular performance by Mg^{2+} in our study are not clear. There is a correlation between Mg^{2+} and coronary artery function where Mg^{2+} stimulates prostaglandin release (Nadler, Goodson, and Rude 1987), which increases coronary flow rate and decreases coronary artery resistance (Bergman et al. 1981). However, the beneficial effects of Mg^{2+} administration in the current study were unrelated to the changes in the coronary perfusion rate. Furthermore, in the current study, there were no changes in ECG parameters, suggesting that the underlying mechanisms by which Mg^{2+} improved cardiac dysfunction in chronic diabetic model was unrelated to its effect on the cardiac electrical remodelling. It is however well known that the Mg^{2+} has an anti-arrhythmic effect (James 2009, Miyoshi et al. 2000). In addition, Mg^{2+} is also an essential factor for sodium-potassium ATPase, which is the main enzymatic activity for intracellular potassium transport during action potential period (Abbott and Rude 1993, Parikka et al. 1999).

The current study showed no morphological changes in the diabetic heart. The gross heart weight was unaltered, and the histological studies showed no evidence of structural changes in the cardiomyocyte cell size or interstitial fibrosis. These findings are in agreement with previous studies. Mihm et al. (2001) documented that there were diastolic and systolic dysfunction occurred in the time course of chronic DM, but without histological evidence of cardiac hypertrophy or fibrosis. In addition, Litwin et al. (1990) showed that there was a cardiac dysfunction without histological alteration in the myocardium, and there was no evidence of interstitial fibrosis in chronic STZ-induced diabetic rats. In contrast, previous studies in chronic STZ-induced diabetic rats showed that there was a cardiac dysfunction with histological evidence of cardiomyocyte hypertrophy and fibrosis (Matyas et al. 2015, Radovits et al. 2015). In addition, the study by Akula et al. (2003) showed that there was LV diastolic and systolic dysfunction based on echocardiography performed at 12 weeks of STZ-induced diabetic rats. However, the lack of histological alterations in our study supports the concept that the nature of diabetic ventricular dysfunction is functional rather than structural.

Although hypomagnesemia is a common metabolic disorder in DM (Chetan P. Hans 2002, Rude 1992), our study showed no changes in plasma Mg^{2+} concentration. Hypomagnesemia has been reported in long term uncontrolled DM (Nagase 1996, Sjogren, Floren, and Nilsson 1986). However, the underlying mechanism of hypomagnesemia in DM is incompletely understood. Osmotic diuresis has reported for a part of Mg^{2+} loss in DM (de Valk 1999, Djurhuus et al. 2001). Unaffected plasma Mg^{2+} level in our study did not rule out the occurrence of hypomagnesemia in our diabetic model, because we did not evaluate Mg^{2+} concentration at tissue level and even at the intracellular compartment.

Limitation of the study and future studies

One limitation of this study is that, although the STZ-induced chronic diabetic complications are known to be similar to those in humans (Wei et al. 2003), there is debate regarding verification of the beneficial effects of Mg^{2+} in clinical trials. Another limitation is to do with the use of Langendorff perfusion experiments and this set up isolates hearts from the whole body and disturbs the normal hormonal and neuronal

cardiac innervation. Furthermore, the underlining mechanisms of the beneficial effects of Mg^{2+} in this study need further clarification.

Further investigations are required to evaluate the precise mechanisms of Mg^{2+} effects. Although our western blot study showed a decrease in the synaptophysin cardiac innervation a further study of other related markers such as hyperpolarization-activated channel (HCN1) expressed in the sinoatrial node, and postsynaptic nerve innervation marker such as PSD95. Furthermore, further metabolic studies to evaluate the involvement of oxidative stress and AGEs will provide a good clarification of the role of Mg^{2+} in diabetic cardiovascular complications.

Conclusion

The results of this study showed that Mg^{2+} administration prevented diabetes-induced cardiac autonomic dysfunction and impairment of cardiac function in chronic DM. However, the overall effects of Mg^{2+} were independent on the hyperglycaemia or plasma Mg^{2+} level. The improvement in autonomic dysfunction by Mg^{2+} in chronic diabetes could have possibly occurred through the preservation of the cardiac autonomic synaptic integrity. The mechanism through which Mg^{2+} improved ventricular dysfunction seemed to be unrelated to the effect of Mg^{2+} on gross cardiac morphological and histological structural changes, electrical remodeling, or coronary perfusion. The clinical implications of our findings are that Mg^{2+} treatment may have long-lasting beneficial effects on the cardiac autonomic impairment and ventricular dysfunction in chronic diabetes. Further studies will need to investigate the detailed underlying mechanisms.

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Appendices

Appendix 1: Animal Ethics Committee approval letter

UNIVERSITY OF CAPE TOWN



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06 May 2014

AEC REF NO: 014/014

Dr A Gwanyanya
Human Biology
Anatomy Building

Dear Dr Gwanyanya

PROJECT TITLE: DIABETES MELLITUS AND THE HEART: EFFECTS OF MAGNESIUM AND OTHER ION CHANNEL MODULATORS ON DIABETES AND MYOCARDIAL INJURY IN DIABETIC WISTAR RAT HEARTS

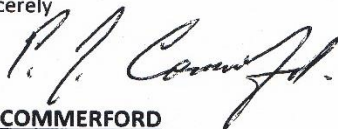
Thank you for submitting your study to the Faculty of Health Sciences Animal Ethics Committee for review.

It is a pleasure to inform you that the FHS AEC has authorised your study specifically for the use of 252 Wistar rats for the period of three years.

Please note that the first annual progress report is due in May 2015.

Please quote the REC REF in all your correspondence

Yours sincerely



PROF PJ COMMERFORD
CHAIR, HSF AEC



Appendix 2: STZ preparation

1- Preparation of the STZ vehicle (citrate buffer)

A) 0.1M citric acid: weigh 0.210 g of citric acid monohydrate and dissolve in 10 ml of distilled water.

B) 0.1M sodium citrate: weigh 0.294 g of sodium citrate tribasic dehydrate and dissolve in 10 ml of distilled water.

Prepare citrate buffer (10 ml) by mixing 4.7 ml of 0.1M citric acid with 5.3 ml 0.1M sodium citrate. Check the pH and adjust it to 4.5 where is not there.

2- STZ preparation and injection

Calculate the amount of STZ required for each rat (50 mg/kg body weight). STZ powder placed in foil covered Eppendorf tube (STZ is a light sensitive). Mix 1 ml of citrate buffer with 50 mg STZ and keep in ice after preparation.

Using 1ml syringe and 24-G needle inject the STZ solution intraperitoneally (within 15 minutes after STZ preparation) per the weight of each rat. For example, rat weighting 250 g, it needs 12.5 mg STZ therefore inject with 0.25 ml of STZ. The control rats were injected with citrate buffer in equal amount of the STZ. The rats were closely observed for two hours after STZ injection.

Appendix 3: Histological protocol

1: Tissue preparation and fixation

After heart isolation, the heart washed shortly about 2 minutes with Krebs solution to washout the blood, then sliced transversely into four slices about 2 mm. The heart slices then fixed in 10% phosphate buffered formalin for 48 hours then transferred into new container containing 70% alcohol for storage until all the samples collected to process together.

2: Tissue processing

The fixed heart tissues were processed using an automated tissue processing system (Leica TP1020, Leica Biosystems, Germany). The heart slices placed in a plastic cassata, labelled then placed in the system, and the program P2 among systems program was used for tissue processing. The automated processing system works for 22 hours for processing the tissues and includes the following stages:

A: Dehydration: the tissues were dehydrated with serial increasing concentration of alcohol as follow:

70% alcohol: 2 x 1hour

96% alcohol: 2 x 1hour

Absolute alcohol: 2 x 1hour

B: Clearing with xylene: 2 x 1hour

C: Impregnation with melted paraffin wax: 2 x 1hour.

3: Tissue embedding

Immediately after tissue processing, the tissue slices placed in metal block and embedded with melted wax using a wax melting system (WD-4, SA). The block was then cooled on a cold plate (CPL-4, Axel Jonson Lab system, SA).

4: Sectioning

The tissue slices in wax block were sectioned into 4- μ m thickness sections with a rotatory microtome (Leica RM2125RT, Leica Biosystems, Germany). The wax block was first firmly clamped in the block holder, then align the wax block with knife, sectioning was done with turning of hand wheel. The section was lifted with un-toothed forceps and transferred into dish containing 30% alcohol, after that the section transferred into distilled water bath at 42°C (Leica HI 1210, SA). Finally, the sections were lifted in slide, and the slides allowed to dry then putted in the oven to melt the paraffin wax before staining.

5: Staining

A- Haematoxylin and eosin (H&E) staining technique

1- De-wax in xylene: 2 x 5 minutes.

2- Hydration: in serial decreasing concentration of alcohol

Absolute alcohol: 3 x 2 minutes. 96% alcohol: 2 x 2 minutes.

70% alcohol: 1 x 2 minutes.

3- Rinse in running tap water: 1 x 1 minute.

4- Stain with haematoxylin: 5 minutes.

5- Rinse in running tap water: 1 minute.

6- Mordant in Scott's tap water: 1 minute.

7- Rinse in running tap water: 1 minute.

8- Stain with eosin: 4 minutes.

9- Rinse shortly in running tap water: < 10 seconds.

10- Dehydration: in a serial increasing concentration of alcohol (briefly < 5seconds with agitation).

70% alcohol: 2 x < 5 seconds. 96%: 2 x < 5 seconds.

Absolute alcohol: 2 x < 5 seconds.

11- Clearing in xylene: 2 x < 10 seconds and the slides lifted in second xylene until ready to be mounted.

12-Section mounting: with dibutyl phthalate –DPX, and place the coverslip over the tissue and the excess DPX removed carefully.

13- Leave the slides to dry at room temperature.

Expected results: blue: nucleus, pink: cytoplasm, erythrocyte, muscle, and collagen fibres.

B- Masson's trichrome staining technique

1- De-wax in xylene: 2 x 5 minutes.

2- Hydration: in serial decreasing concentration of alcohol.

Absolute alcohol: 3 x 2 minutes. 96% alcohol: 2 x 2 minutes.

70%: 1 x 2 minutes.

3- Rinse in distilled water: 1 x 1 minute.

4- Mordant the slides in Bouin's Solution overnight at room temperature.

5- Rinse the slides in running tap water to remove the yellow colour then with distilled water.

6- Stain in Celestine blue: 5 minutes then rinse briefly with running tap water.

7- Stain with haematoxylin: 5 minutes.

8- Mordant in Scott's tap water: 1 minute.

9- Rinse in running tap water: 1 minute.

10- Stain with Biebrich Scarlet- acid fuchsin solution: 5 minutes.

11- Rinse in distilled water: 1 minute.

12- Decolourize with 2.5% phosphotungstic acid-phosphomolybdic acid solution: 5 minutes.

13- Stain with aniline blue solution: 5 minutes.

14- Rinse in distilled water: 1 minute.

15- Enhance differentiation in 1% acetic acid solution: 3 minutes.

16- Rinse in distilled water: 1 minute.

17- Dehydration, clearing, and mounting steps same as that for H&E staining.

Expected results: black: nucleus, blue: collagen fibres, and red: cytoplasm and muscle.

Appendix 4: Western blot protocol

- | | |
|---|--|
| 1- Preparation of buffers | 2-Sample preparation |
| 3- Protein Quantification | 4- Gel preparation and electrophoresis |
| 5-Transfere process | 6- Blocking of the membrane and antibody probing |
| 7- Enhanced Chemiluminescence (ECL) detection | |
| 8- Stripling and re-probing | 9- Scanning and analysis |

1- Preparation of buffers

The following buffers are required for western blot analysis:

A- Radioimmunoprecipitation assay buffer (RIPA buffer): recipe for 50 ml include:

NaCl (5 molar) 1.5 ml, Triton X -100 (100%) 500 ul, SDS (10%) 500 ul, Tris (1molar, Ph 7.5), Deoxycholate sodium 0.5 g and distilled water 46.5 ml.

B- Phosphate Buffered Saline-Tween (PBS-T×1): 8 g NaCl, 0.2 g KCl, 1.44 g

Na₂HPO₄, 0.24 g KH₂PO₄, 2 ml Tween 20 and up to 1000 ml distilled water, stir for 20 minutes and adjust the pH to 7.4.

C- Running (tank) buffer: using a Bio-Rad buffer (10× Tris/Glycine/SDS TGS Buffer, Bio-Rad SA). Mix 100 ml of TGS with 900 ml of distilled water.

D- Transfer buffer: using Bio-Rad buffer (Trans -Blot Turbo ×5 Transfer buffer, Bio-Rad SA). Mix 200 ml of 5× transfer buffer with 600 ml of distilled water and 200 ml of ethanol.

E- Stripling buffer: 8 g NaOH in 1 liter distilled water.

2- Sample preparation

A- Retrieve the frozen right atrial tissues from -80°C freezers. The tissue weighed and placed in new labelled Eppendorf tube and calculate the amount of RIPA extraction buffer (weigh of tissue in gram ×19), this gives the volume of RIPA needed

to add to tissue before homogenisation. Add protease and phosphatase inhibitor cocktail (Halt protease & phosphatase inhibitor, ThermoScientific, USA) 2 ul of cocktail inhibitor per 1 ml of RIPA buffer.

B- Tissue homogenisation by sonication (Soniprep 150, UK). The Eppendorf tube with tissue and RIPA inside are placed in fetched place in the sonicator and allow the Soniprep probe just touch the tissue inside the Eppendorf and sonicate at 180 watts for 20 seconds until all tissue get homogenised in the buffer.

C- Centrifuge the samples in a centrifuge (Labnet International, NJ07095 USA), at 15 000 relative centrifugal force at 4°C for 30 minutes then transfer the supernatant into well labelled Eppendorf tube and make aliquot of 20 ul for protein assay and store at -80°C.

3- Protein quantification

BCA protein assay using the Pierce protein assay kit (Thermo Scientific, Rockford, USA).

A- Preparation of diluted bovine serum Albumin (BSA) standards using RIPA buffer as diluent and standard Bovine serum solution as follows:

Vial	volume of diluent (ul)	volume of BSA(ul)
A	0	300 of stock
B	125	375 of stock
C	325	325 of stock
D	175	175 of vial B dilution
E	325	325 of vial C dilution
F	325	325 of vial E dilution
G	325	325 of vial F dilution
H	400	100 of vial G dilution
I	400	0

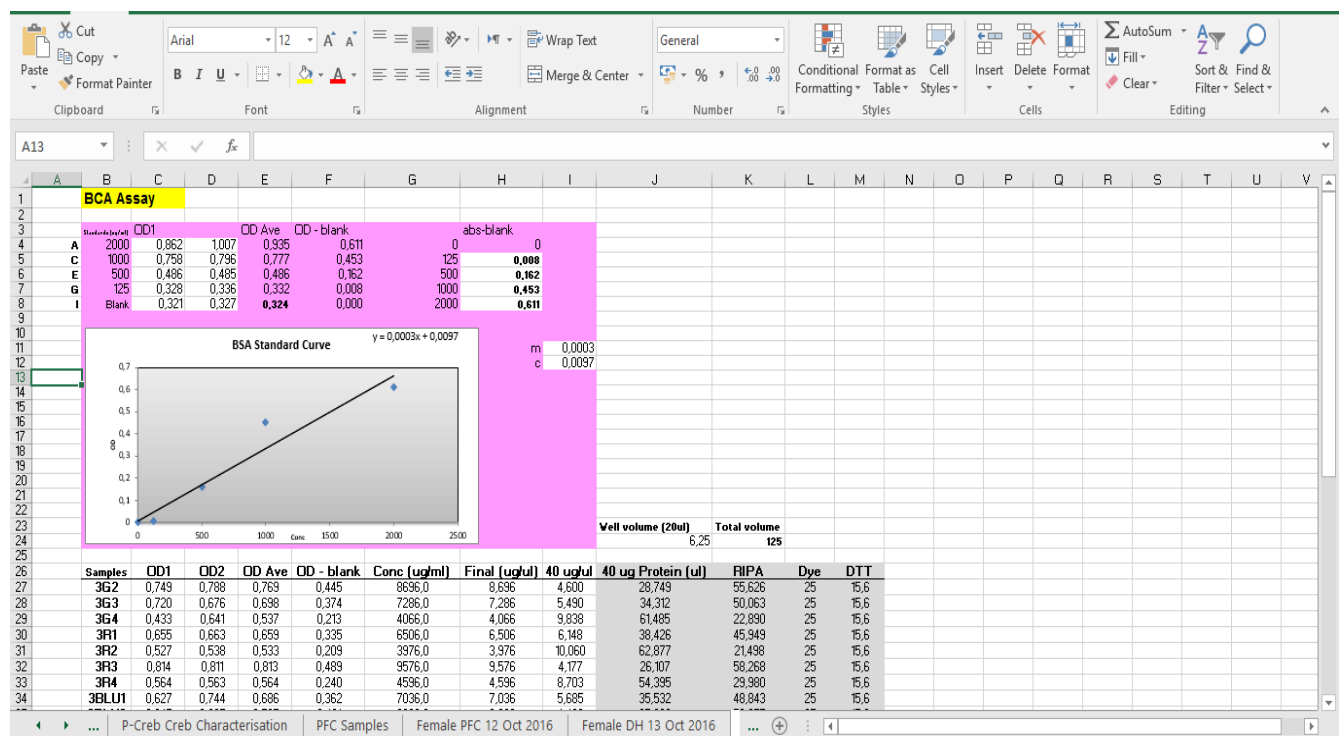
B- Preparation of BCA working reagent

To calculate the amount of working reagent required use the following formula:

Number of standard used + number of samples $\times 2$ (for duplicate) +1 for pipetting error $\times 200$ (amount required per well). To prepare WR mix 50 parts of reagent A and one part of reagent B giving a clear green solution.

C- For a microplate procedure using a flat bottom 96 wells plate, pipette 25 μl of standards in duplicate, 10 μl of samples in duplicate. Add 50 μl of RIPA buffer to the samples and 200 μl of working reagent to both standards and samples. Shake the microplate briefly and cover with parafilm and incubate in the oven at 37°C for 30 minutes.

D- Measure the absorbance in plate reader (RT-2100C, Microplate Reader, Germany) at 562 nm. Create a standard curve and calculate the volume of protein sample required and other recipe, which include RIPA buffer, Lamlli dye (Bio-Rad, SA) and diethyltritroil as shown below:



4- Gel preparation and electrophoresis

Prepare 12% SDS -PAGE gels using ready prepared solution for gel casting (TGX Fast Cast Acrylamide Kit, 10% Cat. #161-0173, Bio-Rad SA). Prepare resolving and stacking gel (note to add 10% Ammonium persulphate (ASP) and Tetramethylethylenediamine (TEMED) just before use. Align glass plates and fix them well in the casting stand and load the resolving gel until about 1 cm below the green line of the casting stand. Add layer of 20% SDS or 100% isopropanol to allow polymerization without oxygen for 10 minutes then remove the layer of SDS. Load the stacking gel and insert the comb to form wells. Allow the gel to set for 30 minutes in room temperature. The hand casted gel can be stored at 4°C for later use.

The prepared samples denatured by boiling at 95°C for 4 minutes then stored in aliquot at -20°C. Before loading the samples were re-boiled at 95°C for 5 minutes, then vortex and centrifuge shortly. Place the gel into electrophoresis chamber and fill with tank buffer and remove the comb carefully from gel and wash the wells with tank buffer. Load 20 µg of samples and molecular weight marker into wells and record the order of loading. Using Mini-PROTEIN Tetra Cell System (Bio-Rad, SA), Connect the electrodes red to red and black to black and switch on the power and run constant voltage at 150 voltage for 90 minutes.

5- Transfer process

Transfer protein from gel into membrane using semidry transfer unit (Trans-Blot Turbo Transfer system, Bio-Rad, SA). Activate Polyvinylidene fluoride (PVDF) transfer membrane (Immuno-Blot PVDF Membrane for Protein Blotting, Bio-Rad, SA) in 100% methanol for 30 seconds then in transfer buffer. Prepare sandwich transfer carefully, pre-wet set of filters paper / PVDF membrane / gel / pre / wet set of filters paper and remove bubble using a roller. Place the sandwich carefully into transfer cassette and close it, then placed in transfer unit and switch on. Choose List / Bio Rad / low molecular weight protein 5 minutes / run. Remove the membrane from transfer unit and rinse briefly with PBS-T. Check transfer of the protein by staining the membrane for 5 minutes with ponceau stain (light sensitive, mix 2.5 ml

of stain with 22.5 ml distilled water). Wash the membrane to remove stain three times with distilled water then block the membrane.

6- Blocking and antibody probing

Block the membrane for one hour at room temperature on the shaker with 5% non-fat milk in PBS-T (dissolve 25 g milk powder in 500 ml PBS-T and filter it with filter paper (Whatman, Germany)). After blocking, incubate the membrane with primary antibody (mouse monoclonal anti-synaptophysin ab18008 Abcam, SA) at a dilution of 1:5000 in 5% non-fat milk overnight at 4°C on shaker. Next day wash in PBS-T 3 times 10 minutes each, then incubate with secondary antibody (Goat Anti-Mouse IgG HRP conjugate, #170-6516, Bio-Rad, SA) at dilution 1:10000 in 5% non-fat milk solution. Incubate with secondary antibody for two hours at room temperature with gentle agitation on shaker. Afterthought washes in PBS-T three times.

7- ECL detection

Prepare detection reagents (Clarity Western ECL Substrate, Cat. #170-5060, Bio-Rad, SA) mix 1ml of each one, first white bottle which is super signal stable peroxide then brown bottle which is super signal west Pico Luminal / Enhancer. Use forceps to remove membrane from PBS-T and place it in a clean dry transparency. Drop the prepared ECL detection solution on the membrane. After one minute, close the transparency and remove excess fluid. Stick a fleshy sticker on the protein marker where expect the protein band signal to appear then keep it detection cassette. In a dark room cut x-ray film (Agfar Healthcare, SA), and lay it on the membrane (without removing the transparency) for 1-3 minutes. Take the x-ray film and immerse it in the developer solution and remove it once the band signal coming up Rinse briefly in water then immerse it in the fixer solution for 2 minutes then rinse in water and hang-up to dry.

8- Stripling and re-probing of the membrane

The purpose of stripling of the membrane is to incubate it with other antibody to detect other protein or for loading control. Immediately after ECL detection process wash the membrane shortly with PBS-T then with distilled water 2 times for 10 minutes each. Wash with stripling buffer for 5 minutes then in distilled water two times for 10 minutes each finally wash briefly with PBS-T.

Block the membrane with 5% non-fat milk for 1 hour at room temperature then incubate with housekeeping primary antibody (Monoclonal Anti-alpha-tubulin antibody produced in mouse, T5168, Sigma -Aldrich, SA) at dilution of 1:3000 in 5% non-fat milk in PBS-T overnight at 4°C. Wash with PBS-T 3 times 10 minutes each. Incubate with horseradish peroxidase (HRP) conjugated secondary antibody (Goat Anti-Mouse IgG-HRP conjugate, # 170-6516, Bio-Rad, SA) at room temperature on the shaker. Wash three times with PBS-T 10 minutes each and proceed to ECL detection as that for protein of interest.

9- Scanning and analysis

Scan the x-ray film with scanner and analyse with image J software (NIH, USA) as following steps:

- 1- Open scanned image in image J software. Change the image to 8-bit mode, go to image / type / 8bit.
- 2-Using rectangular tool from the image J toolbar draw rectangle shape around the first band, then go analyse / gel / select first lane, then drag the rectangle over the next band, go analyse / gel / select next lane, repeat this step until all bands are selected.
- 4- Once all the bands selected go analyse / gel / plot lanes to give a profile plot for each lane. The profile plot represents the mean density of each lane; higher peak represents dark band while wider peak represents large band size. Choose the straight-line tool from image J toolbar and draw a line at the base of the peak to enclose the peak. Select wand tool from image J toolbar and click inside the peak and highlight all the peaks.

5- Choose analyse / gel / label peaks, the results are expressed as a percentage of the mean density of all the bands representing the protein of interest. Copy the result to excel datasheet.

6- Same steps (1-6) needed for the housekeeping protein.

7- Calculate the relative density of each band by dividing the percentage value of the mean density of the protein of interest (synaptophysin) over the percentage value of the mean density of the protein of housekeeping protein (alpha tubulin).